THE INFLUENCE OF TEMPERATURE UPON THE RATE OF REACTION (HÆMOLYSIS, AGGLUTINATION, PRECIPITATION).[1]

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It becomes more and more evident each year, as we advance in the field of immunity, that the bodies which are concerned follow in their action the same laws as other chemical bodies, and therefore must be examined by the methods of modern chemistry. One important step toward the simplifying of this study was made by Ehrlich, when he defined substances of which the action could be measured by test tube experiments. This method allows us to vary the experimental conditions to such an extent that we have control over a whole series of different factors (temperature, dilution, medium, etc.). It is a common observation of all those workers who have investigated hæmolysis, agglutination, precipitation, etc., that these are not simple phenomena and the important bearing which such studies have upon the whole theory of immunity makes it necessary that careful investigation be carried out upon the factors which influence them. Among the factors which play a rôle in chemical reactions, few equal temperature in importance. In the study of hæmolysins and agglutinins it was very early recognized that their activity to a great extent depended upon temperature, increasing as the temperature increased, but diminishing or disappearing at low temperatures.

On account of the great theoretical and practical interest which the study of the dependence of the rate of reaction upon the temperature presents, we have carried out a series of

1 See Toxines et Antitoxines : L'influence de la température sur la vitesse de réaction. I, par Madsen et Walbum; II, par Madsen et Noguchi. *Académie Royale des Sciences et des Lettres de Danemark, Séance du 20 Mai, 1904.*

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experiments with different substances. The experiments include a certain number of hæmolysins (alkalis, acids, bacteriolysins, snake venoms) and some agglutinins and precipitins of different kinds. The ordinary physico-chemical method used for this purpose would be as follows: To determine the rate of reaction, for example, between a hæmolysin and the erythrocytes at different temperatures, and then find the relation between these rates of reaction and the corresponding temperatures. However, this method was not applicable to this investigation, since as yet we do not know the nature of the process which takes place when the hæmolysins and erythrocytes are brought together, and therefore lack the formulas which express these phenomena. For this reason it became necessary to employ another method. The procedure will perhaps be more easily understood by the detailed description of one experiment.

The object of the experiment was to determine the hæmolytic action of sodium hydrate at different temperatures. Seven water-baths with constant temperatures of 39.3°, 34.3°, 30.2°, 27°, 21.2°, 16°, 13.7° C. were prepared. Seven stands, each with six test tubes containing eight cubic centimeters of a one per cent. emulsion of erythrocytes of the horse were put in the baths. When the erythrocytes had been allowed to subside in order to avoid too quick mixture, and the fluid had obtained the surrounding temperature, the amounts of 0.2*n* sodium hydrate indicated in Table I were added as fast as possible (by three experimenters working simultaneously); the tubes were then shaken. Ten minutes afterwards all the stands were taken up at once. It was found that the degree of hæmolysis in the third tube at 39.3° C. (containing 0.06 c.c. of 0.2*n* sodium hydrate) corresponded to a stage intermediate between the second and third tube at 34.3° C. (0.1 c.c.–0.08 c.c.), but nearer the third, and it was estimated that hæmolysis in the third tube at 39.3° would correspond to that caused by .085 c.c. at 34.3° C. In a similar way it was estimated to correspond at 30.2° to 0.11 c.c., at 27° to 0.12 c.c., at 21.2° to 0.22 c.c., at 16° to 0.4 c.c., and at 13.7° to 0.55 c.c.

These results are collected in Table II under the heading 10*m*. In the first column are given the temperatures, in the second

($\frac{1}{c}$ obs.) the observed values, in cubic centimeters, producing the same degrees of hæmolysis. It is obvious that a much smaller amount of sodium hydrate is required at high than at a low temperature to produce the same effect. In the case of a great number of chemical substances, Arrhenius found that the increase of the rate of reaction with increasing temperature could be expressed by the following formula:

$$\frac{c_1}{c_2} = e^{R\left(\frac{T_1 - T_2}{T_1 T_2}\right)}$$

In the formula c_1 and c_2 indicate the concentrations (rates of reaction) at the temperatures T_2 and T_1; μ is a constant, T_1 and T_2 the absolute temperatures, and R, expressed in calories, is 2. The experiment which has been described, like most of our experiments with hæmolysins and agglutinins, can be expressed by this formula. The values calculated in this way are given in the tables under $\frac{1}{c}$ calc. Below is indicated the constant μ in calories. As a rule the observed values correspond with the calculated within the error of experiment.

The difference in the hæmolytic action at low and high temperature expressed by the constant μ is most marked immediately after the addition of the blood emulsion and decreases with increasing time. The final effect—the highest which can be obtained with the hæmolytic substance under the given conditions (medium concentration of blood corpuscles)—is the same for all temperatures; but at low temperatures a much longer time is of course required. The constant μ decreases with increasing time, following apparently a hyperbolic curve, and is zero when the final hæmolytic effect is obtained. Theoretically it would be most correctly determined for the time, zero. This being impossible it must be extrapolated from the other values.

The two *strong alkalis*, sodium and potassium hydrate, of which the degree of dissociation is about the same, act in almost the same manner (Tables II and III). On the contrary there is a marked difference in the case of ammonium hydrate (Table IV). Corresponding to the much smaller rate of reaction of this body, the value of μ is in the beginning much larger, being about double that for sodium and potassium hydrate.

The *final effect* of all alkalis is the same as is obvious from Tables V, VI, and VII. These tables present the hæmolysis produced by *equivalent* amounts of different bases and acids. The erythrocytes were suspended in physiological salt solution or in isotonic cane sugar solution. The numbers 15, 30, 60, etc., indicate the time in minutes required for the production of total hæmolysis. The rate of reaction is the same with equivalent amounts of sodium, potassium, lithium, and barium hydrates, but is much smaller with ammonium hydrate. But if the reaction is increased by shaking the tubes and keeping them some time at 37° C., the difference is diminished and the final limit between the minimal effect and no effect will be about the same. In the tables this limit is marked by a horizontal line. It is obtained after the tubes have been (1) observed six hours at the temperatures indicated in the table; (2) shaken; (3) kept twenty hours at 35.5° C.

The investigation of the hæmolysis produced by *acids* is rendered rather difficult by the strong agglutination and the considerable alteration of the hæmoglobin to which they give rise. Therefore it is difficult to get regular values, but the data given are sufficient to demonstrate the very great differences in the rates of reaction of different acids. Their final effect is nearly the same, but it is often much obscured by agglutination which causes precipitation of the erythrocytes before they are dissolved. This is perhaps one of the reasons why the limit for the "weak" acids is with a little greater amounts than with the "strong" acids. Constant shaking of the tubes with acetic acid will put the limit for this acid at a level with that of sulphuric or hydrochloric acid.

It is interesting to note that both for acids and for alkalis the final limit is obtained with about equivalent quantities, generally a little smaller in the case of alkalis. It is evident that the action of these substances is not directly dependent on the ions. The phenomenon recalls that of the formation of a salt. Acids with a very low degree of dissociation (boric acid) have no hæmolytic action.

Measuring the hæmolytic power of the *acids*, for the above

mentioned reasons, is much more difficult than measuring that of alkalis. If to a series of test tubes, containing equal quantities of a one per cent. emulsion of erythrocytes of the horse, varying amounts of an acid (for example, sulphuric acid) be added, the following is observed: The higher doses will produce a strong brown discoloration of the erythrocytes which clump together and subside, having lost part of their color. With smaller doses total hæmolysis occurs and the color remains almost unchanged. Still further down in the series a decreasing hæmolysis and partial agglutination is observed. Smaller doses produce agglutination alone without hæmolysis.

The rate of action of the readily dissociated acids is so fast that it can hardly be measured. The organic acids generally present less difficulty. We have examined the four lowest members of the fatty acids, namely, *formic*, *acetic*, *propionic*, and *butyric acid*. From Tables VIII, IX, X, and XI, it is obvious that with these acids also the observed values correspond to the calculated. There is a marked difference between formic acid and the three other acids, the constant μ for the former being much smaller and corresponding to the greater rate of reaction. With similar results we have examined *maleinic*, *citraconic*, and *itaconic acid*. In this instance also the acid with the lowest degree of dissociation, the itaconic acid, presents the highest constant, but here too no direct proportion between the rate of reaction and the constant of dissociation is found (Tables XII, XIII, and XIV). Furthermore our investigations include some other hæmolytic bodies: *oleinic acid*, *sodium-oleate*, and *triolein*. Tables XV, XVI, and XVII show that these bodies follow the general rule.

In the above experiments the influence of the temperature has been investigated at different stages of the reaction (ten, twenty, thirty, etc., minutes after its beginning). Some experiments have been carried out, in which only a single stage, suitable for the measurement, has been taken. In this way have been examined: The dissolving effect of *streptolysin* upon the red blood corpuscles of the horse (Table XVIII); the agglutinating action of *ricin* upon rabbit blood (Table XIX); the precipitation produced by strong *sulphuric acid* in a solution of albumen

(Table XX); the agglutination of horse blood by *sublimate* (Table XXI); and the precipitating action of a *specific egg-precipitin* upon egg-albumen (Table XXII). The agglutinating action of *coli-agglutinin* is highly accelerated by increasing the temperature, as the high initial constant of Table XXIII shows. But the longer the time that elapses after the beginning of the reaction, the less the difference observed and the final effect of a given amount of agglutinin is the same at all temperatures. Table XXIV gives an example of a phenomenon which is rather often observed in experiments of this kind: The constants of the measurements, made ten, twenty, and thirty minutes after the beginning of the reaction, decreased regularly; but they subsequently increased very much in the determinations made after forty and fifty minutes. The first group of experiments were carried out in the forenoon, the last mentioned some hours later. Perhaps during this interval a considerable change had occurred in the emulsion of coli-bacilli, of such nature that the influence of the temperature had become much more important. The *agglutinin of Bacillus typhosus* seems in this respect to be quite analogous to the coli-agglutinin (Table XXV).

Among the bacterio-hæmolysins the *vibrio-lysin* (Table XXVI) seems to follow the general rule.[3] Our first experiments with *tetanolysin* seemed to indicate that this body follows the same rule within the temperatures 35.3° C. and 10.8° C. (Table XXVII). This tetanolysin was the same which had served for all the previous experiments in this Institute. In former experiments irregularities of action had never been observed, except when the lysin had become very weak. Now the greatest effect was not produced in the tubes containing the highest quantity of lysin, but in those with the lysin in much weaker concentration. Some examples of this behavior will be found in the following experiments: To twenty-seven tubes, with one per cent. horse blood amounts of lysin decreasing from 1 c.c. to 0.0025 c.c. are added (Table XXVIII). After ten minutes there is almost com-

[3] This vibrio-lysin is made with a vibrio, for which we are indebted to the kindness of Dr. Kraus in Vienna; the same is true for the staphylococcus, mentioned below.

plete hæmolysis with 0.1 c.c.; from this tube the hæmolysis decreases in the series both upwards and downwards; almost the same degree of hæmolysis is observed with 1 c.c. and with 0.07 c.c. Three and a half hours later the series presents the following result: Total hæmolysis with 0.1 c.c. and 0.07 c.c. The tubes with smaller quantities show a gradually decreasing hæmolysis. In the tubes with higher amounts of lysin the hæmolysis is strongest with 0.13 c.c. gradually decreasing to a minimum at 0.3 c.c. and 0.25 c.c.; from this point upwards the hæmolysis again increases, and with 1 c.c. almost total hæmolysis occurs. After a time all the tubes containing over 0.1 c.c. presented total hæmolysis, and therefore we would not have had any idea of these great irregularities in the rate of reaction if we had made our examination only on the following day.

These irregularities are not due to the weakness of this lysin, as we have found similar irregularities with quite fresh cultures. Thus Table XXIX shows an experiment in which hæmolysis at 90° C. and 16° C. advances regularly; H_{10}, H_{20}, H_{40} indicate the time in minutes required for total hæmolysis. But at 26 C.° is found a broad zone between 0.25 c.c. and 0.05 c.c in which total hæmolysis occurs after ten minutes, and upward and downward in the series decreasing lysis is found. At 40° C. after ten minutes total hæmolysis occurred in the tubes with 0.8, 0.5, 0.3, 0.2, 0.08 c.c. and in all the tubes from 0.04 to 0.015 c.c. In the intermediate tubes the hæmolysis was very much less.

As all the experiments with this lysin presented similar results, it was extremely difficult to compare the effect at different temperatures. There seems to exist a maximum around 31.7° C. while the action is much smaller at other temperatures. Some examples are given in Tables XXX and XXXI. In several series two values are given indicating the presence of the above mentioned irregularities.

It might be thought that the decrease of the hæmolytic action at higher temperatures, 36.5° and 41.7° C., might possibly be due to a weakening of the tetanolysin at these temperatures. However some experiments, carried out specially to settle this question, showed another curious phenomenon: in a certain

number of cases the activity of the lysin was increased considerably by a short heating to 37° C. As an example the following experiment is given. The broth medium usually employed (0.5 per cent. glucose, 2 per cent. pepton, 1 per cent. sodium chloride) was inoculated February 2 with tetanus bacilli and kept under paraffin at 35.5° C. until February 21. It was then taken from the incubator and kept at low temperature, about 4° C. Three days afterwards it was heated to 37° C. and the hæmolytic effect then tested. In Table XXXII $\frac{1}{c}$ indicates those amounts of the culture, heated to 37° C., during the time t, which have the same action. The experiments have been carried out during a series of successive days and have shown that the increase of the hæmolytic power is sometimes rather considerable. Other tetanus cultures present analogous phenomena. The experiment described by Table XXXIII shows how heating to 37° C. during ten minutes can quadruple the hæmolytic power of the culture.

We have, however, obtained several times tetanus cultures without these irregularities and the influence of the temperature has conformed to the usual rule.

Another lysin, *staphylolysin*, showed the peculiar phenomenon described above, the highest hæmolytic effect not occurring at the highest temperature. A given quantity produced the maximal action between 20° C. and 30° C., the effect being considerably smaller at higher or lower temperatures (Table XXXIV).

With another body, which has played an important rôle in the hæmolytic experiments of recent years, *lecithin*, hæmolytic action does not seem to depend very much on temperature. Table XXXV presents observations after 20, 30, 50, 60, 90, 140, and 330 minutes at four different temperatures, namely, 0.1°, 11.1°, 24.05°, and 36.7° C. The figures in columns indicate hæmolysis in percentages.[4] The process proceeds rather regularly; the effect seems to be about the same at 0.1° C. and 36.7° C. and a little less at the intermediate temperatures.

In the case of *cobra venom* there is a little increase of hæmolysis

[4] Arrhenius and Madsen: Physical Chemistry Applied to Toxins and Antitoxins, Festskrift ved Indvielsen af Statens Seruminstitut, Copenhagen, 1902.

at higher temperature; a minimum seems to exist between 14° C. and 24° C. (Table XXXVI). On the contrary hæmolysis by the venom of *Ancistrodon piscivorus* (water moccasin) is considerably greater at low than at high temperature (Table XXXVII). It is interesting to remember that this venom is used by the water moccasin to poison chiefly cold-blooded animals.

The experiments which have been communicated must be considered only as preliminary steps into a field which has not yet been investigated; owing to the great errors of experiment and to our still very incomplete methods of measurement the data cannot be definitive. A striking feature is, that among a number of experiments which have corresponding results are found irregular series, which do not follow the usual course. In such instances it seems reasonable to think that influences of catalytic nature have had a part, increasing or decreasing the rate of reaction.

Our experiments show that hæmolysis, agglutination, and precipitation by a great many different substances increase with increasing temperature, following the same formula, which applies to other chemical phenomena:

$$\frac{c_1}{c_2} = e^{\frac{\mu}{R}\left(\frac{T_1 - T_2}{T_1 T_2}\right)}$$

This is true for the hæmolysis by the hydrates of sodium, potassium, and ammonia, by formic, acetic, propionic, butyric, maleinic, citraconic, itaconic, and oleinic acids, by sodium oleate and by triolein, and furthermore by streptolysin and vibrio-lysin. It is true for the agglutination of the erythrocytes by ricin and mercuric chloride, for the precipitation of albumen by sulphuric acid and by specific precipitin; and also for the bacterial agglutination by the agglutinins of Bacillus coli and Bacillus typhosus. Still the increase of the action with the temperature can only reach a certain limit, partly owing to the destruction of the medium (erythrocytes, bacteria), partly owing to the weakening of the active body itself (bacteriolysin, agglutinin).

On the contrary, the tetanolysin showed peculiarities, for example, a maximum activity at 32° C.; staphylolysin showed

a maximum activity between 20° C. and 30° C.; with lecithin and cobra venom hæmolysis at low and high temperature seems to proceed with about equal rapidity and to be more rapid than at the intermediate temperatures; the venom of the water moccasin acts most strongly at low temperatures.

TABLE I.

ACTION OF 0.2*n* NaOH UPON 1 PER CENT. EMULSION OF HORSE BLOOD AT DIFFERENT TEMPERATURES.

	39.3°	34.3°	30.2°	27.°	21.2°	16°	13.7°
1	0.1 c.c.	0.13 c.c.	0.2 c.c.	0.2 c.c.	0.3 c.c.	0.8 c.c.	0.8 c.c.
2	0.08 "	0.1 "	0.17 "	0.17 "	0.25 "	0.6 "	0.6 "
3	0.06 "×	0.08 "×⁒	0.13 "	0.13 "×*0.2	"×⁒	0.5 "	0.5 "
4	0.05 "	0.06 "	0.1 "×⁒	0.1 "	0.17 "	0.4 "	0.4 "
5	0.04 "	0.05 "	0.08 "	0.08 "	0.13 "	0.3 "	0.3 "
6	0.03 "	0.04 "	0.06 "	0.06 "	0.1 "	0.25 "	0.25 "

* Corresponds to an amount between two that are given but nearer the larger.

× Corresponds to an amount midway between two that are given.

⁒ Corresponds to an amount between two that are given but nearer the smaller.

TABLE II.

0.2 *n* NaOH.

10*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
39.3	0.06	0.057
34.3	0.085	0.084
30.2	0.11	0.12
27.0	0.12	0.155
11.2	0.22	0.26
26.0	0.4	0.4
13.7	0.55	0.5

μ = **15190**

20*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
39.3	0.055	0.054
34.3	0.07	0.069
30.2	0.085	0.086
27.0	0.1	0.1
21.2	0.13	0.14
16.0	0.185	0.184
13.7	0.26	0.21

μ = **9486**

30*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
34.3	0.06	0.057
30.2	0.07	0.071
27.0	0.065	0.083
21.2	0.115	0.116
16.0	0.15	0.15
13.7	0.19	0.17

μ = **9441**

40*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
39.3	0.04	0.04
34.3	0.05	0.05
30.2	0.1	0.063
27.	0.07	0.073
21.2	0.1	0.1
16.0	0.13	0.13
13.7	0.17	0.15

μ = **9210**

50*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
38.5	0.043	0.042
34.3	0.048	0.049
30.8	0.07	0.057
26.8	0.065	0.067
21.1	0.07	0.085
15.8	0.1	0.107
14.6	0.115	0.112

μ = **7415**

60*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
34.3	0.045	0.045
30.8	0.055	0.052
26.8	0.065	0.061
21.1	0.07	0.077
15.8	0.09	0.096
14.6	0.1	0.12

μ = **7185**

80*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
38.5	0.035	0.038
34.3	0.045	0.044
30.8	0.055	0.05
26.8	0.055	0.059
21.1	0.085	0.074
15.8	0.07	0.091
14.6	0.1	0.095

$\mu = 6907$

100*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
38.5	0.04	0.045
34.3	0.05	0.05
30.8	0.06	0.058
26.8	0.07	0.067
21.1	0.085	0.081
15.8	0.085	0.098
14.6	0.1	0.1

$\mu = 6263$

180*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
38.5	0.04	0.04
34.3	0.04	0.045
30.8	0.057	0.05
26.8	0.06	0.057
21.1	0.07	0.068
15.8	0.07	0.081
14.6	0.085	0.085

$\mu = 5663$

TABLE III.

0.2*n* KOH.

10*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
38.8	0.11	0.1
35.7	0.14	0.12
28.8	0.17	0.18
25.4	0.21	0.23
19.5	0.33	0.34
15.3	0.45	0.45
13.4	0.5	0.52

$\mu = 11710$

20*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
38.1	0.065	0.076
35.9	0.085	0.084
28.5	0.17	0.12
25.4	0.15	0.14
19.4	0.2	0.195
15.2	0.225	0.245
14.1	0.275	0.26

$\mu = 9210$

30*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
38.1	0.1	0.096
35.9	0.13	0.11
25.4	0.17	0.17
15.2	0.25	0.28
14.1	0.3	0.29

$\mu = 8288$

40*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
38.6	0.06	0.06
28.6	0.085	0.09
25.4	0.12	0.11
19.4	0.13	0.14
15.2	0.17	0.17
13.7	0.18	0.18

$\mu = 7968$

60*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
38.1	0.07	0.076
35.9	0.085	0.081
28.5	0.1	0.1
25.4	0.13	0.12
19.4	0.1	0.14
15.2	0.17	0.17
14.1	0.17	0.173

$\mu = 6078$

120*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
35.6	0.05	0.053
28.4	0.065	0.064
25.3	0.07	0.07
19.4	0.09	0.084
15.1	0.09	0.095
14.5	0.1	0.097

$\mu = 5164$

180*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.8	0.04	0.04
35.6	0.037	0.044
29.3	0.05	0.051
25.6	0.06	0.055
20.3	0.065	0.062
15.3	0.07	0.07
13.8	0.07	0.073

$\mu = 4145$

TABLE IV.

0.5*n* NH_4OH.

10*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.5	0.04	0.043
34.8	0.085	0.083
29.7	0.17	0.17
25.9	0.3	0.3
21.0	0.60	0.64

$\mu = 26760$

20*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.2	0.03	0.029
34.8	0.05	0.05
30.2	0.085	0.081
25.7	0.19	0.17
21.0	0.3	0.32
15.7	0.55	0.69

$\mu = 24360$

30*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.5	0.0275	0.0225
34.8	0.035	0.039
29.7	0.07	0.072
25.9	0.13	0.12
21.0	0.25	0.225
15.2	0.5	0.5
11.0	0.7	0.89

$\mu = 23020$

40*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.1	0.02	0.019
34.6	0.03	0.031
30.7	0.05	0.048
25.9	0.085	0.086
21.3	0.17	0.15
16.1	0.3	0.3
12.2	0.45	0.49

$\mu = 21640$

60*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.2	0.019	0.014
34.8	0.024	0.022
30.2	0.035	0.035
25.7	0.05	0.056
21.1		
15.7	0.17	0.17
12.2	0.26	0.26

$\mu = 19150$

80*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.2	0.025	0.02
34.8	0.03	0.03
30.2	0.04	0.046
25.7	0.07	0.069
21.1	0.1	0.11
15.7	0.18	0.18
12.2	0.3	0.26

$\mu = 16660$

100*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.1	0.02	0.018
34.6	0.025	0.025
30.7	0.025	0.034
25.9	0.045	0.051
21.3	0.06	0.075
16.1	0.115	0.12
12.2	0.17	0.17

$\mu = 14920$

120*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.1	0.017	0.017
34.6	0.0225	0.0225
30.7	0.0275	0.029
25.9	0.04	0.04
21.3	0.06	0.055
16.1	0.1	0.079
12.2	0.17	0.1

$\mu = 13720$

180*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.0	0.015	0.015
34.7	0.0185	0.0185
30.9	0.0225	0.0225
25.9	0.03	0.029
21.5	0.04	0.037
16.1	0.05	0.05

$\mu = 9441$

TABLE V.

BLOOD OF HORSE 1% (0.9% NaCl). Temperature 16.2°.

Amount in c.c.	Hydrochloric acid m/1	Sulphuric acid m/2	Formic acid m/1	Acetic acid m/1	Butyric acid m/1	Oxalic acid m/2	Succinic acid m/2	Lactic acid m/1	Tartaric acid m/2	Ammonia m/1	Sodium hydrate m/1	Potassium hydrate m/1	Lithium hydrate m/1	Amount in c.c.	Barium hydrate m/4
0.25										60				0.5	
0.2				15†										0.4	
0.17							15							0.34	
0.13										120				0.26	
0.1														0.2	
0.07				30§				15		210	15	15	15	0.14	15
0.05				60§			30 60§		15†		30	30	30	0.1	30
0.04	15*			120				30			60	60	60	0.08	60
0.03		15†	15			15†		60	30§					0.06	
0.025	30*	30	30	450		30 60	120† 450†	120† 45o	60	450†	120	120	120	0.05	120
0.02	60*	60	60			450			120		210	210	210	0.04	
0.017	120§ 450	120* 450	120† 450			120§			450		450	450	450†	0.034	210† 450
0.013														0.026	
0.01														0.02	
0.007														0.014	
0.005														0 01	

* Corresponds to an amount between two that are given, but nearer the larger.
† Corresponds to an amount midway between two that are given.
§ Corresponds to an amount between two that are given but nearer the smaller.

TABLE VI.

BLOOD OF HORSE 2.5% (0.9% NaCl). Tp. 15.2°.

Amount in c.c	Hydrochloric acid $\frac{m}{1}$	Sulphuric acid $\frac{m}{2}$	Acetic acid $\frac{m}{1}$	Mono- $\frac{m}{1}$	Di-chlor-acetic acid $\frac{m}{1}$	Tri- $\frac{m}{1}$	Oxalic acid $\frac{m}{2}$	Succinic acid $\frac{m}{2}$	Lactic acid $\frac{m}{1}$	Citric acid $\frac{m}{3}$	Ammonia $\frac{m}{1}$	Sodium hydrate $\frac{m}{1}$
1.0			5 15†									
0.7											30	
0.5								15 30				
0.4			30						5		60	
0.3												
0.25											120	
0.2										60†		
0.17												5
0.13							5	60 120	15 30			
0.1	5 15 30	5 15 30		5			15 30					15
0.07			60	15	5							30
			120§		15†	5 15				120		
0.05	120† 60	120† 60		30					60 120			60
0.04				120† 60	60† 30	60† 30	60† 120					120
0.03					120	120						
0.025												
0.02												
0.017												
0.013												
0.01												
0.007												

For explanation of signs *, †, and § see Table V.

TABLE VII.

BLOOD OF HORSE 1% (7.8% CANE SUGAR). Tp. 14.6°. 0.9% NaCl.

Amount in c.c.	Hydrochloric acid m/1	Sulphuric acid m/2	Formic acid m/1	Acetic acid m/1	Di-chlor-acetic acid m/1	Tri-acetic acid m/1	Butyric acid m/1	Oxalic acid m/2	Succinic acid m/1	Lactic acid m/2	Tartaric acid m/2	Citric acid m/3	Ammonia m/1	Sodium hydrate m/1	Hydrochloric acid m/1	Ammonia m/1	Sodium hydrate m/1
1.5									5			14					
1.3									14		5						
1.0									60† 30		14	30	90*	5			
0.7									90		30	60		14			
0.5									240			90†				30	
0.4				5†							60			30			
0.3				60* 14							90	240				60	
0.25				240										60			
0.2										5† 14			240§			90	
0.17	5									30* 60				90			
0.13	14		14† 5							90†	240						5
0.1			60											240§		240	14
0.07	30	14†	240†					5 14							5		30
0.05		30						30§							14		
0.04	60 90	60						60							30		60
0.03	240	90			5 14	30		240							60		90*
0.025		240			90 240	240									90		240
0.02																	
0.017																	
0.013																	
0.01																	
0.007																	
0.005																	
0.004																	
0.003																	
0.0025																	
0.002																	
0.0017																	

For explanation of signs *, †, and § see Table V.

TABLE VIII.

FORMIC ACID (0.1 *n*).

10*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
37.0	0.17	0.16
34.5	0.175	0.18
29.9	0.185	0.227
24.5	0.32	0.3
20.0	0.4	0.364
15.0	0.45	0.483
10.45	0.6	0.62

μ = 8841

20*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
37.0	0.13	0.128
34.5	0.13	0.142
29.9	0.17	0.17
24.5	0.21	0.214
20.0	0.225	0.26
15.0	0.35	0.33
10.45	0.4	0.405

μ = 7598

30*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
37.0	0.13	0.13
34.5	0.13	0.135
29.9	0.17	0.15
24.5	0.185	0.173
20.0	0.19	0.194
15.0	0.225	0.221
10.45	0.25	0.252

μ = 4310

40*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
37.0	0.13	0.132
34.5	0.13	0.136
29.9	0.15	0.146
24.5	0.17	0.157
20.0	0.175	0.168
15.0	0.18	0.181
10.45	0.19	0.195

μ = 2578

50*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
37.0	0.13	0.128
34.5	0.13	0.133
29.9	0.14	0.142
24.5	0.16	0.156
20.0	0.18	0.168
15.0	0.18	0.183
10.45	0.185	0.198

μ = 2901

180*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
37.0	0.12	0.12
34.5	0.125	0.122
29.9	0.125	0.125
24.5	0.125	0.129
20.0	0.13	0.132
15.1	0.135	0.132
10.4	0.14	0.14

μ = 921

TABLE IX.

ACETIC ACID. $\frac{1}{1}n$

10*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
40.2	0.025	0.0209
34.7	0.035	0.0405
30.1	0.065	0.073
24.7	0.145	0.147
19.6	0.30	0.29
15.1	0.45	0.532

μ = 23580

15*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
40.2	0.02	0.0175
34.7	0.03	0.033
30.1	0.04	0.057
24.7	0.1	0.11
19.6	0.225	0.212
15.1	0.4	0.375

μ = 22240

20*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
40.2	0.02	0.0152
34.7	0.025	0.0251
30.1	0.035	0.0364
24.7	0.055	0.067
19.6	0.115	0.115
15.1	0.25	0.183
10.2	0.3	0.316

μ = 18050

40*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
40.2	0.017	0.0131
34.7	0.02	0.0203
30.1	0.025	0.0298
24.7	0.033	0.047
19.6	0.065	0.074
15.1	0.13	0.11
10.2	0.17	0.18

$\mu = 15520$

50*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
40.2	0.017	0.013
34.7	0.02	0.0203
30.1	0.025	0.0274
24.7	0.03	0.0417
19.6	0.05	0.063
15.1	0.1	0.091
10.2	0.13	0.141

$\mu = 14230$

60*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
40.2	0.017	0.0114
34.7	0.017	0.0164
30.1	0.0225	0.0229
24.7	0.03	0.0338
19.6	0.04	0.0467
15.1	0.075	0.07
10.2	0.1	0.105

$\mu = 13210$

90*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
40.5	0.017	0.0134
35.15	0.017	0.017
30.4	0.0225	0.0213
24.7	0.0285	0.0281
19.6	0.035	0.0364
14.5	0.04	0.047
10.1	0.06	0.06

$\mu = 8752$

120*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
35.15	0.017	0.0164
30 4	0.02	0.02
24.7	0.025	0.0253
19.6	0.03	0.03
14.5	0.033	0.0392
10.1	0.05	0.0481

$\mu = 7459$

210*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc
40.5	0.015	0.0147
35.15	0.016	0.0169
30.4	0.02	0.0193
24.7	0.024	0.0226
19.6	0.0275	0.0262
14.5	0.03	0.03
10.1	0.033	0.0352

$\mu = 5112$

TABLE X.

PROPIONIC ACID. $\frac{I}{I}$ *n*.

10*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.15	0.0275	0.0267
35.25	0.04	0.0455
28.45	0.09	0.112
23.6	0.275	0.35
19.35	0.4	0.4

$\mu = 24860$

20*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.15	0.025	0.0176
35.25	0.0275	0.0253
28.45	0.04	0.0483
23.6	0.07	0.079
19.35	0.14	0.11
14.9	0.30	0.20
7.8	0.40	0.44

$\mu = 18050$

30*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.2	0.02	0.0172
35.15	0.022	0.0238
28.45	0.04	0.042
23.45	0.05	0.067
19.55	0.09	0.095
14.8	0.20	0.149
7.7	0.30	0.30

$\mu = 15890$

40*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.2	0.017	0.0152
35.15	0.02	0.0207
28.45	0.033	0.0353
23.45	0.043	0.055
19.55	0.065	0.076
14.8	0.15	0.116
7.7	0.25	0.226

$\mu = 15060$

60*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.2	0.017	0.0116
35.15	0.02	0.0153
28.45	0.025	0.025
23.45	0.03	0.037
19.55	0.04	0.0505
14.8	0.06	0.078
7.7	0.185	0.136

$\mu = 13720$

90*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.1	0.017	0.0164
35.15	0.02	0.0192
28.8	0.025	0.025
23.4	0.027	0.0317
19.35	0.04	0.0379
14.9	0.042	0.047
8.0	0.085	0.0775

$\mu = 7690$

120*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.1	0.017	0.017
35.15	0.02	0.02
28.8	0.025	0.0227
23.4	0.025	0.0265
19.35	0.03	0.03
14.9	0.033	0.034

$\mu = 5065$

180*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.1	0.017	0.017
35.15	0.017	0.019
28.8	0.0225	0.0223
23.4	0.025	0.0258
19.35	0.033	0.029
14.9	0.035	0.033
8.0	0.04	0.04

$\mu = 4789$

TABLE XI.

BUTYRIC ACID. $\frac{I}{I}n$.

10*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
37.0	0.03	0.03
34.4	0.033	0.04
30.7	0.07	0.062
24.35	0.13	0.13
19.5	0.25	0.25
15.9	0.28	0.38

$\mu = 21550$

20*m*

Tp.	$\frac{I}{I}$ obs.	$\frac{I}{c}$ calc.
34.4	0.025	0.025
30.7	0.04	0.038
24.35	0.065	0.076
19.5	0.15	0.13
15.9	0.20	0.2

$\mu = 19890$

30*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
37.0	0.02	0.018
34.4	0.0225	0.0225
30.7	0.025	0.031
24.35	0.048	0.052
19.5	0.08	0.08
15.9	0.1	0.11
10.3	0.2	0.19

$\mu = 15190$

40*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
37.0	0.018	0.016
34.4	0.020	0.02
30.7	0.022	0.027
24.35	0.03	0.043
19.5	0.065	0.065
15.9	0.10	0.086
10.3	0.13	0.14

$\mu = 14000$

50*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
37.0	0.017	0.016
34.4	0.019	0.019
30.7	0.025	0.025
24.35	0.028	0.04
19.5	0.045	0.058
15.9	0.085	0.076
10.3	0.12	0.12

$\mu = 13210$

TABLE XII.

MALEINIC ACID. $\frac{I}{I}n$.

10*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
37.5	0.014	0.014
34.7	0.017	0.017
29.9	0.03	0.0235
24.7	0.04	0.034
19.3	0.05	0.05
15.8	0.065	0.065
12.2	0.07	0.086

$\mu = 12670$

20*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
37.5	0.014	0.013
34.7	0.015	0.0145
29.9	0.017	0.018
24.7	0.022	0.024
19.3	0.03	0.032
15.8	0.04	0.038
12.2	0.045	0.047

$\mu = 9118$

30*m*

Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
37.5	0.013	0.012
34.7	0.013	0.013
29.9	0.017	0.016
24.7	0.018	0.021
19.3	0.025	0.027
15.8	0.03	0.032
12.2	0.04	0.038

$\mu = 8281$

TABLE XIII.

CITRACONIC ACID. $\frac{I}{I}$ *n*.

10*m*			20*m*			50*m*		
Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
35	0.017	0.017	35	0.013	0.012	38	0.0115	0.0115
30.2	0.02	0.025	30.2	0.017	0.017	35	0.013	0.012
23.7	0.04	0.04	23.7	0.025	0.026	30.2	0.015	0.015
20.3	0.05	0.051	20.3	0.03	0.032	23.7	0.017	0.016
15.6	0.085	0.076	15.6	0.047	0.045	20.1	0.017	0.017
9.8	0.1	0.098	9.8	0.065	0.068	15.6	0.019	0.019
μ = 13490			μ = 11690			μ = 4375		

TABLE XIV.

ITACONIC ACID. $\frac{I}{I}$ *n*.

15*m*			20*m*		
Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
29.8	0.03	0.03	29.8	0.025	0.024
24.2	0.05	0.051	24.2	0.0375	0.039
17.8	0.085	0.096	17.8	0.05	0.069
14.6	0.15	0.13	14.6	0.09	0.093
10.7	0.2	0.2	10.7	0.135	0.135
μ = 17040			μ = 15560		

TABLE XV.

OLEINIC ACID. $\frac{I}{10}$ *n*.

10*m*			22*m*		
Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
39.4	0.035	0.035	39.1	0.03	0.03
36.7	0.06	0.05	36.2	0.035	0.042
31.4	0.1	0.1	31.3	0.08	0.079
23.9	0.3	0.3	24.1	0.2	0.2
μ = 25780			μ = 23480		

TABLE XVI.

OLEATE OF SODIUM. $\frac{1}{100}$ *n*.

10*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
36.3	0.125	0.125
31.4	0.14	0.14
24.1	0.18	0.16
15.9	0.19	0.19
12	0.2	0.21
4	0.25	0.25

$\mu = 3776$

TABLE XVII.

TRIOLEIN. $\frac{1}{100}$ *n*.

15*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
38.9	0.22	0.22
35.7	0.25	0.25
30.9	0.27	0.36
24	0.6	0.6

$\mu = 13810$

50*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
38.9	0.17	0.17
35.7	0.2	0.2
30.9	0.32	0.26
24	0.4	0.4

$\mu = 10590$

TABLE XVIII.

STREPTOLYSIN.

20*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
36.1	0.08	0.08
31.1	0.20	0.21
25.8	0.40	0.54
22.9	1.30	1.15

$\mu = 31880$

TABLE XIX.

RICIN.

c. 30*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
36.2	0.05	0.05
27.9	0.12	0.11
15.6	0.33	0.37
10.1	0.70	0.66
0.9	1.30	1.80

$\mu = 17220$

TABLE XX.

10 *n* H_2SO_4.

2% EGG ALBUMEN.

90*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
35.8	0.1	0.11
29.7	0.16	0.16
25.4	0.225	0.21
19.9	0.3	0.29
14.5	0.4	0.41

$\mu = 11050$

TABLE XXI.

0.1 *n* $HgCl_2$

RED CORPUSCLES OF HORSE.

45*m*

Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
35.8	0.085	0.085
30.9	0.15	0.15
25.9	0.25	0.24
21.9	0.55	0.36
16.1	0.6	0.65
12.3	1.0	0.98
10.8	1.0	1.15

$\mu = 17860$

TABLE XXII.

PRECIPITIN.

73*m*

Tp.	$\frac{I}{C}$ obs	$\frac{I}{C}$ calc.
36.1	0.25	0.25
30.1	0.3	0.306
20.0	0.45	0.44
13.9	0.55	0.55

$\mu = 6284$

TABLE XXIII.

COLI-AGGLUTININ.

20*m*

Tp.	$\frac{I}{C}$ obs.	$\frac{I}{C}$ calc.
41.2	0.0025	0.0023
36.6	0.004	0.0042
31.4	0.0115	0.0086
25.8	0.030	0.019
19.4	0.05	0.05
15.6	0.06	0.092
12.3	0.085	0.15

$\mu = 25960$

30*m*

Tp.	$\frac{I}{C}$ obs.	$\frac{I}{C}$ calc.
41.2	0.0007	0.00063
36.6	0.001	0.0011
31.4	0.001	0.0021
25.8	0.0017	0.0044
19.4	0.012	0.011
15.6	0.018	0.018
12.3	0.026	0.029

$\mu = 23850$

50*m*

Tp.	$\frac{I}{C}$ obs.	$\frac{I}{C}$ calc.
41.2	0.0004	0.00035
36.6	0.0005	0.00056
31.4	0.0005	0.00098
25.8	0.0007	0.00184
19.4	0.002	0.0038
15.6	0.0055	0.0061
12.3	0.01	0.0093

$\mu = 20030$

65*m*

Tp.	$\frac{I}{C}$ obs.	$\frac{I}{C}$ calc.
40.9	0.00018	0.000175
36.7	0.0002	0.00022
30.7	0.0005	0.00038
25.9	0.0005	0.00062
19.4	0.0008	0.00116
15.9	0.002	0.0017
12.0	0.002	0.0025

$\mu = 17080$

80*m*

Tp.	$\frac{I}{C}$ obs.	$\frac{I}{C}$ calc.
41.5	0.0002	0.000115
36.7	0.00013	0.00015
30.8	0.00025	0.00023
25.9	0.0002	0.00031
19.5	0.00045	0.00049
15.8	0.0007	0.00064
12.1	0.0017	0.00085

$\mu = 12200$

100*m*

Tp.	$\frac{I}{C}$ obs.	$\frac{I}{C}$ calc.
41.5	0.0001	0.0001
36.7	0.000085	0.000125
30.8	0.000185	0.00017
25.9	0.00019	0.00022
19.5	0.00035	0.00031
15.8	0.0004	0.00039
12.1	0.00045	0.00047

$\mu = 9486$

120*m*

Tp.	$\frac{I}{C}$ obs.	$\frac{I}{C}$ calc.
41.9	0.000085	0.000084
36.7	0.0001	0.0001
30.7	0.00018	0.00014
25.9	0.0002	0.00017
19.4	0.000225	0.00023
15.9	0.000275	0.00027
12.0	0.00033	0.00033

$\mu = 8288$

TABLE XXIV.

COLI-AGGLUTININ.

10*m* I			10 *m* II			20*m* I			20*m* II			30*m*		
Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
38.6	0.005	0.0036	38.6	0.005	0.005	39.5	0.0015	0.0013	39.5	0.001	0.001	38.5	0.001	0.00088
34.9	0.0055	0.0057	34.9	0.0055	0.0088	35.7	0.002	0.002	35.7	0.0015	0.0017	35.0	0.0012	0.0012
30.9	0.01	0.011	30.9	0.02	0.016	31.5			31.5	0.003	0.003	30.7	0.00175	0.0018
24.0	0.04	0.035	24.0	0.05	0.05	24.1	0.01	0.0084	24.1	0.01	0.0088	24.0	0.007	0.0034
21.2	0.045	0.057	21.2	0.085	0.08	20.5	0.0125	0.013	20.5	0.007	0.015	21.6	0.007	0.0044
13.2	0.25	0.25	12.7	0.35	0.35	14.1	0.03	0.039	14.1	0.04	0.041	12.7	0.01	0.011
									10.5	0.048	0.074	9.6	0.015	0.015
	μ = 30950			μ = 29340			μ = 22820			μ = 26380			μ = 17180	

40*m* I			40*m* II			50*m*			5*h*		
Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
38.5	0.0007	0.0007	38.5	0.0006	0.00065	38.5	0.0007	0.00062	38.5	0.0002	0.00018
35	0.00085	0.0012	35.0	0.00125	0.00125	35.0	0.001	0.0011	35.0	0.0002	0.0002
30.7	0.002	0.0022	30.7	0.006	0.0037	30.7	0.002	0.0022	30.7	0.0002	0.00024
24	0.008	0.0063	24.0	0.007	0.0057	24.0	0.009	0.0069	24.0	0.0003	0.00031
21.6	0.011	0.0093	21.6	0.035	0.035	21.6	0.01	0.01	21.6	0.00042	0.00033
12.7	0.04	0.04	12.7	0.04	0.044				12.7	0.00052	0.00049
									9.6	0.00052	0.00055
	μ = 29030			μ = 28320			μ = 30630			μ = 6907	

TABLE XXV.

B. TYPHOSUS-AGGLUTININ.

10*m*			40*m*			120*m*		
Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
38.2	0.002	0.002	38.6	0.0013	0.00126	37.8	0.0006	0.0006
35.2	0.006	0.0036	34.9	0.00225	0.0024	35.2	0.0008	0.00079
31.1	0.007	0.0083	19.6	0.04	0.04	31.3	0.00115	0.00115
27.4	0.02	0.018	16.1	0.1	0.079	27.4	0.0014	0.0018
19.2	0.13	0.09	11.4	0.15	0.2	15.8	0.006	0.006
15.9	0.2	0.2						
11.5	0.5	0.55						
	$\mu = 37160$			$\mu = 33010$			$\mu = 18690$	

180*m*		
Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
37.8	0.0007	0.0007
35.2	0.0009	0.00083
31.3	0.001	0.00107
27.4	0.0013	0.0014
19.0	0.0017	0.0025
11.6	0.0045	0.0044
	$\mu = 8511$	

TABLE XXVI.

VIBRIO-LYSIN.

20*m*			40*m*		
Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc
35.3	0.17	0.1	35.3	0.115	0.078
29.8	0.25	0.25	29.8	0.17	0.17
26.3	0.4	0.4	26.3	0.22	0.27
20.6	1.0	1.0	20.6	0.6	0.6
	$\mu = 27330$			$\mu = 24410$	

100*m*			180*m*		
Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.	Tp.	$\frac{I}{c}$ obs.	$\frac{I}{c}$ calc.
40.1	0.0275	0.0275	39.8	0.03	0.03
35.3	0.05	0.044	35.4	0.042	0.043
29.5	0.07	0.08	29.3	0.07	0.073
26.0	0.1	0.117	25.3	0.1	0.104
20.4	0.25	0.215	20.1	0.17	0.17
16.1	0.42	0.394	15.6	0.3	0.25
	$\mu = 19340$			$\mu = 15840$	

TABLE XXVII.

TETANOLYSIN.

60*m*			65*m*		
Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.	Tp.	$\frac{1}{c}$ obs.	$\frac{1}{c}$ calc.
30.4	0.4	0.37	35.3	0.45	0.45
25.6	0.5	0.53	31.1	0.58	0.58
21.9	0.8	0.66	24.9	0.8	0.83
15.4	1.0	1.0	10.8	2.0	2.1
12.1	1.3	1.27			
μ = 10860			μ = 10860		

TABLE XXVIII.

1.0 c.c.	0.1 c.c.	0.01 c.c.
0.7	0.07	0.007
0.5	0.05	0.005
0.4	0.04	0.004
0.35	0.035	0.0035
0.3	0.03	0.003
0.25	0.025	0.0025
0.2	0.02	
0.17	0.017	
0.13	0.013	

TABLE XXIX.

TETANOLYSIN.

Amount in c.c.	40°	26°	16°	9°
1.3				H_{10}
1.0				
0.8	H_{10}			
0.6				
0.5	H_{10}			
0.4				H_{20}
0.3	H_{10}			
0.25			H_{10}	
0.2	H_{10}			H_{40}
0.17				
0.13		H_{10}		
0.1			H_{20}	
0.08	H_{10}			
0.06			H_{40}	
0.05				
0.04				
0.03	H_{10}			
0.02				
0.015				

TABLE XXX.

TETANOLYSIN.

25*m*		
Tp.		$\frac{1}{c}$
41.7		0.25
36.5		0.6
31.7		0.03
25.7	or	0.07
		0.2
21.2		0.17
14.9		0.2
6.7	or	0.4
		0.8

TABLE XXXI.

Tp.	10m $\frac{I}{c}$	10m $\frac{I}{c}$	20m $\frac{I}{c}$	40m $\frac{I}{c}$	60m $\frac{I}{c}$	90m $\frac{I}{c}$	120m $\frac{I}{c}$
41.7	0.35	0.4	0.185	0.1	0.07	0.05	0.06
36.5	0.55	0.5	0.2	0.1	0.07	0.07	0.045
31.71	or 0.25 / 0.06	or 0.17 / 0.025	<0.03	0.025	0.0275	0.017	0.013
25.7		0.6	0.225	0.07	0.085	0.085	0.0275
21.2	0.8		0.1	0.05		0.06	0.04
14.9	0.18		0.07		0.085	0.06	0.07
6.7	1.7	2.0	0.4	0.17	0.115	0.2	0.085

TABLE XXXII.

TETANOLYSIN.

Heating at 37°.

$^{24}/_{2}$ t	$\frac{I}{c}$	$^{25}/_{2}$ t	$\frac{I}{c}$	$^{26}/_{2}$ t	$\frac{I}{c}$	$^{27}/_{2}$ t	$\frac{I}{c}$	$^{1}/_{3}$ t	$\frac{I}{c}$	$^{3}/_{3}$ t	$\frac{I}{c}$
0m	0.5	0m	0.7	0m	0.225	0m		0m	0.2	0m	0.07
30	0.45	30	0.5	20	0.2	5	0.03	5	0.175	10	0.07
60	0.275	60	<0.1	30	0.03	10	0.05	10	0.15	20	0.07
120	<0.2	120	0.7	40	0.33	20	0.05	20	0.1	30	0.05
		180	0.7	50	0.5	30	0.05	40	0.1	40	0.03
		240	0.7	60	0.5	40	0.07			50	0.03
				80	0.7	50	0.4			60	0.07
				100	0.55	60	0.35			90	0.1
				120	0.7	80	0.3			120	0.1
				180	0.5	120	0.3			180	0.16
				240	0.225	240	0.3			240	0.13
				360	0.3	360	0.3			300	0.06
						480	0.3			360	0.04
						600	0.3			420	<0.02
										480	<0.02

TABLE XXXIII.

NEW CULTURE.

Amount in c.c.	Heated 20 *m.* at 37°	Not heated.
1.0		H_{35}
0.7		H_{75}
0.5		H_{180}
0.4		
0.3		
0.25		
0.2	$>H_{35}$	
0.17		
0.13	H_{75}	
0.1	H_{180}	

TABLE XXXIV.

STAPHYLOLYSIN.

(Blood of Rabbit 1 %.)

15*m*		20*m*		45*m*		60*m*	
Tp.	$\frac{1}{c}$	Tp.	$\frac{1}{c}$	Tp.	$\frac{1}{c}$	Tp.	$\frac{1}{c}$
36.5	0.35	36.4	0.3	36.4	0.35	28.6	0.095
29.1	0.17	28.9	0.17	28.6	0.08	24.4	0.08
24.3	0.225	24.4	0.14	24.4	0.07	19.7	0.08
19.5	0.33	18.8	0.22	19.7	0.075	11.3	0.53
13.6	1.8	12.5	1.55	11.3	0.6		

TABLE XXXV

LECITHIN. $\frac{1}{20}$ *n.*

	20*m*				30*m*				50*m*				60*m*			
	0.1°	11.1°	24.05°	36.7°	0.1°	11.1°	24.05°	36.7°	0.1°	11.1°	24.05°	36.7°	0.1°	11.1°	24.05°	36.7°
0.3	95	100	100	98	99	100	100	100	100	100	100	100	100	100	100	100
0.2	87	—	99.5	95	96	—	—	98	—	—	—	—	—	—	—	—
0.1	76	85	93	90	92	—	98	95	98	—	—	99	—	—	—	—
0.08	65	80	85	87	90	95	96	93	96	—	99	97	—	—	—	98
0.06	50	65	60	85	85	90	90	92	94	95	97	95	97	99	98	97
0.04	25	25	25	80	70	80	75	90	90	88	85	92	95	90	89	95
0.03	0	0	0	75	58	30	60	85	75	65	80	90	90	70	82	92
0.02				40	35	0	0	50	50	30	45	60	60	30	48	65
0.01				25	0			0	20	0	20	20	35	0	20	35
0.008				0					0		0	0	0		0	0

	90*m*				140*m*				330*m*			
	0.1°	11.1°	24.05°	36.7°	0.1°	11.1°	24.05°	36.7°	0.1°	11.1°	24.05°	36.7°
0.3	100	100	100	100	100	100	100	100	100	100	100	100
0.2	—	—	—	—	—	—	—	—	—	—	—	—
0.1	—	—	—	—	—	—	—	—	—	—	—	—
0.08	—	—	—	—	—	—	—	—	—	—	—	—
0.06	—	—	—	—	—	—	—	—	—	—	—	—
0.04	—	95	90	98	—	—	92	—	—	—	—	—
0.03	95	80	85	95	—	90	87	—	—	—	—	—
0.02	85	42	60	70	90	55	70	80	98	70	96	98
0.01	65	0	20	25	68	20	20	35	80	80	25	83
0.008	25		0	0	45	0	0	0	48	0	0	55
0.006	0				0				25			25
0.004									5			0
0.003									0			

TABLE XXXVI.

NAJA TRIPUDIANS.

	5*m*		10*m*		15*m*	
	$\frac{1}{c}$ obs.		$\frac{1}{c}$ obs.		$\frac{1}{c}$ obs.	
Tp.	I	II	I	II	I	II
37·3	0.4	0.3	0.275	0.225	0.275	0.225
35·3	0.4	0.3	0.3	0.25	0.275	0.225
30.8	0.4	0.33	0.35	0.26	0.3	0.25
24.1	0.5	0.33	0.4	0.35	0.4	0.3
17·7	0.4	0.3	0.45	0.35	0.45	0.3
14.2	0.6	0.45	0.5	0.38	0.35	0.25
10.8	0.5	0.4	0.45	0.3	0.3	0.25

TABLE XXXVII.

ANCISTRODON PISCIVORUS.

	5*m*		10*m*		15*m*	
	$\frac{1}{c}$ obs.		$\frac{1}{c}$ obs.		$\frac{1}{c}$ obs.	
Tp.	I	II	I	II	I	II
39·3	0.5	0.38	0.4	0.35	0.4	0.28
35·2	0.5	0.36	0.45	0.35	0.4	0.28
30·7	0.4	0.35	0.42	0.35	0.4	0.3
28.2	0.4	0.35	0.4	0.35	0.45	0.33
19.2	0.4	0.3	0.35	0.28	0.4	0.3
14.8	0.4	0.3	0.35	0.28	0.3	0.25
10.6	0.4	0.25	0.3	0.25	0.25	0.225

THE FORMATION OF TRUE BONE WITH CELLULAR (RED) MARROW IN A SCLEROTIC AORTA.

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At a recent post-mortem examination in the Johns Hopkins Hospital one of the most striking lesions found was an extremely sclerotic and calcified aorta. Microscopical sections from the dorsal portion of this aorta showed at one point in the much thickened intima a new formation of true bone. While the finding is not unparalleled, the references in medical literature to similar lesions are so few that the case seems worthy of note, especially as in one particular at least this case differs from those already reported.

The patient was a white male 72 years old, and was admitted to the surgical service for the removal of a hypertrophied prostate, but died before the operation, apparently as the result of a general infection, following an attack of furunculosis, and of complications as indicated in the anatomical diagnosis. The post-mortem examination was made by the author August 12, 1904, three hours after death. It does not seem necessary for the purpose of this paper to give the full post-mortem findings. The Anatomical Diagnosis was as follows:

Hypertrophy (adenomyoma) of the prostate; dilatation of the bladder with hypertrophy of its musculature; chronic diffuse nephritis; aortic sclerosis with calcification; furunculosis; purulent bronchitis; broncho-pneumonia; mural thrombi of aorta; embolism and thrombosis of the right brachial artery, with gangrene of the right extremity, and of the right internal carotid artery, with encephalomalacia of the right cerebral hemisphere; atrophic spleen; surface atrophy of the liver.

The lesions of interest are those of the aorta and its branches. In addition to the sclerosis, the presence of the thrombi in the

aorta seems noteworthy. The larger of these formed a globular mass about 15 millimetres in diameter, attached to the convex side of the ascending portion of the aorta at a point just below the orifice of the innominate artery. It was firmly adherent to the wall of the vessel. This was quite evidently the source of the emboli which were carried to the right brachial and carotid arteries. A smaller, oval, somewhat flattened thrombus mass capped a roughened, projecting, calcified plaque in the upper portion of the thoracic aorta.

The whole aorta showed extreme sclerosis with calcified plaques throughout its extent. The calcification, however, was most marked in the lower dorsal portion and just above its bifurcation the aorta was a rigid tube with walls of bony hardness. It was through this portion that microscopical sections were made after decalcification in 5 per cent. nitric acid.

The sections gave the following picture on microscopical examination:

The adventitia appears normal, showing no increase in the density of the fibrous tissue. At the ends of the section the media is considerably thinner than normal and is about one third as wide as the thickened intima. The inner portion of the media (that next the intima) has a denser appearance than the outer part. Here its fibres stain more deeply with eosin and are in part of the section distinctly hyaline, with but here and there a shrunken pyknotic nucleus. Throughout the media the nuclei seem to be reduced in number. Toward the middle part of the section the media becomes even thinner, the hyaline degeneration of the inner portion is more extensive, and in many places there has been a deposition of calcium salts in the hyaline area. At the thinnest part of the media, its continuity is almost completely interrupted by the passage obliquely through it of vessels of considerable size, surrounded by granulation tissue, which extends inward into the intima. At this point the muscle fibres are extremely atrophied and the nuclei much shrunken.

The intima is thickened about the whole circumference of the aorta. The thickening is irregular with numerous nodules projecting toward the lumen, the largest of which lies over the point in the media where the muscle fibres are most atrophied. This nodule is of the nature of the so-called atheromatous abscess. Toward the lumen of the aorta there is a fairly well-preserved layer of dense connective tissue, poor in cells. This is in large part hyaline and toward the centre of the nodule shows deposits of calcium salts deeply stained by the hæmatoxylin. The main mass of the nodule is made up of a granular débris, in which are calcium salts and numerous slit-like spaces evidently left by the solution of cholesterin crystals. There are also a few round vacuoles, evidently fat spaces. There has been at one point toward the media an attempt at organ-

zation and repair. One finds here fairly well-formed vascular connective tissue, in which are numerous lymphoid cells as well as larger mononuclear cells which contain as a rule coarse granules of a light yellow pigment. There are in this part of the intima masses of deep red (eosin staining) refractile materail, often surrounded by cells, which are evidently deposits of old hyaline fibrin.

At the base of this atheromatous nodule and lying directly adjacent to the calcified media are two masses of true bone, not connected with each other in any of the sections studied, though lying quite close together. The larger mass is ring-shaped and has a definite marrow cavity. The bone gives indication of a definite laminar structure. It contains numerous typical bone-corpuscles and is penetrated at its thickest portion by a capillary vessel surrounded by a layer of cells resembling osteoblasts closely applied to the wall of bone, the whole appearing to represent a Haversian canal. About this mass of bone there is a layer of cells resembling osteoblasts and forming a definite membrane, while at many places in the sections studied are multinuclear cells of the typical osteoblast type. A few of these lie in lacunar depressions in the bone but many more are found in similar depressions in the adjacent calcified media. The marrow cavity of the bone contains in a delicate reticulum numerous fat cells and between these a few cells of the marrow type; large mononuclear cells, among which a few are phagocytic and contain yellow blood pigment; small mononuclear and polymorphonuclear cells, both neutrophilic and eosinophilic. This marrow, however, is not so cellular as the portion lying between this ring of bone and the second bone mass. This second mass consists of two bars, a slender one lying immediately adjacent to the calcified media and a larger one separated from the first by a small amount of myeloid tissue, containing vessels, fat cells, and large multinuclear giant cells, apposed to the media. These masses of bone are also surrounded by a layer of osteoblasts and contain bone corpuscles.

The tissue lying between these bony masses has the typical appearance of the cellular red marrow of cancellous bone. It is quite richly vascularized by delicately walled capillaries. There are scattered fat cells in the area, and all the types of cells usually found in red marrow, with a tendency to the grouping normally found there. Under the low power one is struck first by the numerous giant cells of the megalokaryocyte type with large twisted polymorphous nuclei. Under higher power one is able to demonstrate mononuclear cells of the myelocyte type, many of which contain eosinophile granules, polymorphonuclear cells both eosinophilic and neutrophilic, lymphoid cells and cells of the erythrocyte series, non-nucleated red cells, normoblasts with their pyknotic nuclei and hæmoglobin-containing cell bodies with sharp contour, and similar cells but with reticular nuclei.

The picture then in brief is that of an aorta with a media much atrophied and showing hyaline degeneration and in places the deposition of calcium salts. The intima is much thickened, shows atheromatous degeneration, and in places the signs either of trauma and hemorrhage or of an inflammatory process (old hyaline fibrin masses and blood pigment in phagocytes). There

is also invasion of the intima by vessels from the adventitia surrounded by young connective tissue cells and mononuclear cells, possibly an attempt at organization following the hemorrhage or inflammation. Where this tissue comes in touch with the calcified tissue there is the formation of true bone with a cellular marrow as found in cancellous bone.

The cases reported in medical literature of bone formation in sclerotic vessels are not numerous, as has been said in a preceding paragraph. Of those reported but six concern the aorta, the two earliest of which were described by Mönckeberg.[1] Marchand[2] in his article in Eulenburg's *Real-Encyclopädie* described a case in which in the crural artery he found the calcified media invaded by new vessels with the absorption of the calcified material and the deposition of the bone by osteoblasts upon the calcium deposits with the formation of a marrow space around the vessel.

Howse[3] in 1877 described a focus of bone in the axillary artery. The axillary artery of the patient had been ruptured by trauma and at his death some time subsequently the artery was found surrounded at the point of injury by a firm mass of connective tissue, in which there was found microscopically cancellous bone "produced in tissue which represents and springs from an altered condition of the middle coat."

In 1886 Paul[4] demonstrated before the London Pathological Society a specimen from a sclerotic tibial artery in which there was a focus of bone formation in the intima.

In the same year Cohn[5] investigated a number of calcified areas in the aorta, without finding anything "even approaching the picture of a bone corpuscle," but did find in a sclerotic and calcified aortic valve a mass of true bone adjacent to the calcified material; and also three cases in which there was bone formation in the media of the crural artery.

In 1900 Rosenstein[6] reported bone formation in one calcified aortic segment, and both bone and cartilage in another leaflet of the same valve.

Rohmer[7] followed in 1901 with three cases, one with bone in the aortic valve, one in the mitral, and one in which there were found several foci of bone in the intima of the femoral artery.

v. Schrötter[8] in 1901 described bone in an organized thrombus in the anterior tibial artery and also in another case in the intima of the crural artery.

The most extensive search for bone formation in sclerosed vessels has been made by Mönckeberg,[1] who in 1902 reported ten successful cases in the study of about 100 cases. In two cases he found bone both in the intima of the aorta and in the media of the femoral artery, in one in the media of the femoral, in one in both media and intima of the femoral, in one case in the intima of the tibialis posticus, and one in the intima and media of both the tibialis anticus and posticus. In this latter case cartilage was formed. He adds in a note to his article that in addition to these six cases he has been successful in four others in finding bone in medium-sized sclerotic vessels, but does not give any further details.

Thorel[9] has found bone formation in the aorta and in the femoral and tibial arteries.

Poscharissky[10] examined fourteen hearts and found bone in three of them, twice in the mitral valve and once in the calcified annulus of the left venous ostium. Of thirty-one vessels examined, four gave positive results, three of which were aortæ, the fourth being the femoral artery. He records further the finding of vascular granulation tissue in vessels in five autopsies.

In all then, bone formation has been found in the aorta six times, in the intima and media of medium-sized arteries twenty-two times, and in heart valves eight times. This cannot represent the frequency of the occurrence of the condition. Virchow[11] had noted the lesion and in his *Cellularpathologie* stated emphatically that, although most of the so-called bony aortæ and vessels were undoubtedly calcified, the formation of true bone does occur, although he cited no specific case. Orth[12] similarly describes in his text-book the formation of bone in the intima of the aorta, again without reference to any special case. Although the author has made no investigation of the frequency of the occurrence of the lesion, Mönckeberg's figures of ten positive results in a hundred cases of marked sclerosis would indicate that the lesion is regarded as rare chiefly because it is not more often searched for.

For an explanation of the presence of these foci of bone in such unexpected places, one would turn naturally to the Cohnheim doctrine of embryonic displacement of tissue, were not the cases so numerous, and did not the figures just quoted show such a frequency of occurrence of the process in advanced arteriosclerosis.

Another argument against such a theory is the evidence offered by the investigation of Pollack,[13] under Lubarsch's direction, of calcified foci in the lungs, as a result of which, of forty-seven cases studied, free bone was found adjacent to the calcium deposits in forty-three. Poscharissky likewise has conducted an extensive search for bone formation in calcified foci in various organs and records seventeen positive findings in the lungs of twenty-eight cases, two in the tonsils, four positive in the lymph glands, twelve in the dura mater, one in a calcified myoma uteri, two positive and one negative in the liver, one in an adrenal

tumor, and one in a hypernephroma of the kidney, besides those in the heart and vessels quoted above.

The final and convincing evidence against the metastasis or displacement theory is afforded by the experimental work of Barth,[14] of Sacerdotti and Frattin, [15] and of Poscharissky. [10] Barth introduced a portion of incinerated bone into the peritoneal cavity of a cat. Six weeks later it was found penetrated by connective tissue and in several places there was found true bone in contact with the calcium-containing material, lined by osteoblasts arranged partly in a regular row and partly in groups, forming an osteoid tissue which passed over without a sharp line into ordinary connective tissue.

Sacerdotti and Frattin tied off the renal vessels on one side in four rabbits. Three months later the animals were killed and in three of them there was found a formation of true bone adjacent to the calcium deposits in the necrotic tissue, with a growth of vascular granulation tissue into the kidney from the capsule. This experiment was repeated by Poscharissky on five rabbits with successful results in three animals. Similar experiments by him on the spleen and ovary in the rabbit were unsuccessful, obviously because there was no capsule in these organs with independent circulation from which granulation tissue might grow.

It seems clear, then, that one has here a metaplasia of connective tissue cells into osteoblasts, for in vessels and heart valves there can be no possibility of periosteal or perichondral sprouts playing a part, although in pulmonary foci such a possibility is not so easily excluded. As to the stimulus which leads to this metaplasia, there seems to be a uniformity of opinion as might be expected from the similarity of the pictures presented by the different cases. The process was clearly stated by Paul as consisting of three steps:

(1) Calcareous degeneration.

(2) Irritation about these places from fracture or other injury leading to inflammatory proliferation.

(3) Ossification in this young proliferating tissue.

In all the cases the bone has been formed where young con-

nective tissue has come into contact with calcium deposits. The metaplasia is apparently the result of the chemical stimulus of the calcium salts. These two factors are accepted by all, but there still seems some question as to the cause for the invasion of the intima of the sclerotic arteries by the new vessels and connective tissue. Paul suggested trauma of the nature of fracture of the calcified plates. This view is supported by Howse's case of rupture of the axillary artery; by one of Cohn's cases, in which there was a rupture of the calcified media of the crural artery; and possibly by the present case, in which there were signs of hemorrhage and old inflammatory exudate in the intima of the aorta. Poscharissky insists that in addition to the calcium salts there is always a deposit between the granules of a material resembling amyloid.

The question as to the mode of the formation of the bone I shall not discuss at length. There is about an equal division of those who have written on the subject between the two views:

(1) That there is a direct metaplasia of connective tissue into bone after the manner of callus formation, and

(2) That the calcified material is eroded with the formation of vascularized spaces containing young connective tissue cells, some of which take on the function of osteoblasts and lay down bone, some of the osteoblasts becoming included and forming the bone corpuscles.

The different cases give evidence that the bone may be formed in either way. In the present case the bone would seem to have been formed by the second method. There was nothing in the section suggesting the formation first of osteoid tissue, then of bone, but instead one found erosion of calcified material being carried on by osteoclasts. There were marrow spaces containing capillary loops, and osteoblasts arranged in a row or membrane adjacent to the bone. The picture was quite that seen at the epiphyseal line, with, however, the calcified media of the aorta in place of the calcified cartilage of the epiphysis. The determination of the exact mode of formation of the bone does not seem of so much interest as is the bare fact that in a person of advanced years the connective tissue cells under the stimulus of the calcium

salts should retain the power of differentiation into osteoblasts. The patient in this case was 72 years of age. The six of Mönckeberg's cases in which the age was specified were between 62 and 84 years. The greater number of Lubarsch's cases were in patients of advanced age, although there were seven under 45 years and one under 10. Poscharissky obtained only negative results in calcified lung foci in four persons under 28 years.

The present case differs from all others thus far reported in which bone was found in the vessels, in the nature of the marrow found in the intima of the aorta adjacent to the bone. In the previous cases the marrow when present was fatty. In this, there was true cellular marrow indistinguishable in section from the marrow of the ribs or of the cancellous bone. Attention was first attracted to it by the rather numerous giant cells with large twisted nuclei distinctly of the megalokaryocytic type. Closer study showed the presence of myelocytes and polymorphonuclear cells, both eosinophilic and neutrophilic, mononuclear and lymphoid cells, and cells of the erythrogenetic group; nucleated cells of the normoblast type and others showing reticular nuclei, as well as many erythrocytes.

The origin of this marrow and its relation to the surrounding tissue seem of the greatest interest. If one excludes the possibility that the bone-formation represents an embryonic displacement, as I think has been done, that explanation can hardly apply to the marrow, and one is left with but two possible explanations: (1) that one has in this also a direct metaplasia of the granulation tissue cells, or (2) that the tissue is the result of transportation of bone marrow cells by the blood stream and their emigration from the blood vessels. Let us consider the latter proposition first. If we accept this view and regard the marrow tissue as the result of emigration of cells we are forced to accept either one of two further propositions. If one be a believer in the Ehrlich idea that in adult life there is no genetic connection between the various types of leucocytes, the granular and the mononuclear (hyaline) groups, or between them and the erythrocytic group, to maintain the emigration idea in this case he must postulate the emigration of a parent cell for each

one of the several types found. This seems on the face of it improbable, as the myelocytes, nucleated red cells, and giant cells are so infrequently found in the circulation in general, and possess so little migratory power. It would seem that one might disregard this possibility as being too remote. The other possibility which is open, if one accepts the emigration theory, is that there has occurred the outwandering of a cell or of cells of an indifferent type, which are capable of differentiating into the various forms of blood cells. The view that such a cell exists in the marrow seems to be gaining adherents among students of hematology, as the result of the writings of Pappenheim, Wolff, Hirschfeld, Naegeli, and others. This cell is a simple undifferentiated cell with large round vesicular nucleus and scanty protoplasm and has been variously called, the large lymphocyte, the indifferent lymphoid cell, the myeloblast, Troje's *Markzell*, etc. Such a cell is found in the cell groups of the marrow and in the germinative centres of the lymph glands. It is found in the circulatory blood under certain conditions, in greatest numbers in acute lymphocytic leukæmia. By an increase in its protoplasm it becomes the large mononuclear cell always found in the circulation. If one grants that such a cell is capable of differentiation into the various types of blood cells in adult life, as is strenuously denied by the Ehrlich school, and as strenuously maintained by others, there is no great difficulty in explaining the marrow focus found in the aorta in question.

It would seem more rational, probably, to assume that a single process was operative in the formation of both the bone and the marrow, that is that they were both the result of metaplasia of the connective tissue cells. The bone corpuscles and marrow cells have a common embryonic ancestor in the undifferentiated mesoblastic cell, although in adult life they seem to have diverged widely in nature and function. While it seems but a short step from fibroblast to osteoblast, merely a question of the nature of the intercellular substance laid down, it seems a much greater transition from the fibroblast to the polymorphonuclear cell with its specific granulation, its highly developed power of motility,

and its power of phagocytosis. Yet it appears (Jackson) that the cells of the marrow in the fœtus are developed from the cells of the original invading periosteal bud, the osteoblasts. So in this case the same stimulus which led to the metaplasia of connective tissue cells into osteoblasts may have determined their further differentiation into marrow cells, or possibly the stimulus may have been the presence of the newly formed bone.

Although this is the only case recorded in which marrow of this type has been found in the blood vessels, it has been found adjacent to bone foci in other organs. Pollack says that whenever bone is formed in the lung, marrow is also formed, though usually it is of the fatty type but showing a few lymphoid cells and cells of myelocytic type. In two cases he found also nucleated red cells. Poscharissky found marrow in every case, but his result is to be accepted only if one accepts his definition of marrow, for he considers the cellular granulation tissue described in every case marrow, and says that usually in old cases it becomes fatty marrow. He also, however, found in some cases erythroblasts, megalokaryocytes, osteoblasts, and myelocytes. Gierke [16] has also described in a calcified adrenal in a $3\frac{1}{2}$-year-old girl, the formation of bone with a marrow focus containing giant cells, myelocytes, and polymorphonuclear cells and eosinophiles.

The case then here reported is not unparalleled, and it would seem that one must accept the view that the marrow focus was the result of metaplasia of cells of the young granulation tissue in the environment of the newly-formed bone. There is but one point which prevents these cases from being absolutely positive evidence of the formation of blood cells from connective tissue cells in adult life, and that is the presence in young granulation tissue of round cells, mononuclear cells of doubtful antecedents but identical in form and staining characteristics with cells of the blood and marrow. Pappenheim, among others, insists that the whole group of round cells and plasma cells found in granulation tissue are histogenetic (as opposed to hæmatogenetic) in origin, but emphasizes in a recent communication the fact that indifferent lymphoid cells (histogenetic) are found

in every granulation tissue. The extensive and careful researches of Maximow led him to the conclusion that the cells of the mononuclear group found in granulation tissue were emigrated cells from the blood stream, and thus arise either from marrow or lymph-glands, and for this view there seems much evidence. The embryonic type of these cells and their similarity to if not identity with cells of the blood and marrow would suggest that the marrow arose from a metaplasia of them, rather than of the fixed connective tissue cells of adult type, and upon the settling of the question of their origin depends the decision in the case here recorded as to whether the marrow focus is the result of metaplasia of connective tissue cells or of metaplasia of indifferent cells of lymphoid type which have emigrated from the blood stream. However that question may be decided, the cases do show that cells of the connective tissue and blood groups retain into late life embryonic characteristics and are capable of a diverse development under appropriate stimuli, and further that there is a much closer genetic relation between the types of blood cells in adult life than is generally accepted.

I would conclude from the study of this case and the literature bearing on the subject, that:

(1) Metaplastic formation of bone in sclerotic vessels is not a rare phenomenon.

(2) The factors in its formation are extensive sclerosis with the presence of calcium deposits; traumatic or inflammatory disturbance in the calcified area, with penetration of the same by granulation tissue; the formation of bone adjacent to the calcified areas.

(3) The bone may be deposited in two ways: either by the conversion of connective tissue into a callus or osteoid tissue with subsequent conversion into bone and at times with the formation of cartilage; or by the resorption of calcified material by vessels and osteoclasts, the formation of a marrow space, and the deposit of bone in its periphery by an osteoblastic membrane derived by metaplasia from the connective tissue cells accompanying the vessel.

(4) In the present case, in which all of the elements of true

cellular marrow were found, one is not justified in deciding whether they were formed by a further metaplasia of connective tissue cells or by the metaplasia of emigrated cells from the blood stream, capable of differentiation into the various types of marrow cells.

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THE RELATION OF TYPES OF DIARRHŒA IN CHILDREN TO STRAINS OF BACILLUS DYSENTERIÆ.[1]

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The association of the dysentery bacillus with diarrhœa in infants is now established. Previous studies on the subject from this laboratory having proven this occurrence, we undertook as our present thesis the separation from the dejecta of all bacteria which could be cultivated, and which agglutinated with the patient's blood, exhibited pathogenic properties for laboratory animals, or belonged to the so-called dysentery group of bacilli. A part of our problem, to which we gave particular attention, was the determination of the special type, strain, or variety of dysentery bacilli occurring in the different classes of cases of diarrhœa, and the frequency with which these strains were found associated with the cases.

The entire number of infants studied was seventy-four. They could be divided clinically into the several groups which follow with the number of cases of each group studied.

Inflammatory colitis,—29 cases.

Toxic, dyspeptic, or fermental diarrhœa,—36 cases.

Intestinal indigestion,—7 cases.

Chronic gastro-enteritis,—1 case.

Tuberculous ileo-colitis,—1 case.

In twenty of the cases malnutrition was a marked feature. It was thought unwise to attempt further to subdivide the cases. It is doubtful if our series included any of the nervous diarrhœas given in the classification suggested by Dunn.[2] The result of treatment was as follows: Thirty children recovered completely, twelve improved, one was unimproved, and thirty-one died.

[1] This study was carried out with the aid of a grant from the Rockefeller Institute for Medical Research.

[2] Dunn, *Archives of Pediatrics*, 1905, xxii, 401.

In order that a comparison could be made between the types of clinical disease and the bacteriological findings this series of cases was further subdivided according to the results of the bacteriological studies. The subdivisions employed were the following:

A. Cases in which the Shiga type of dysentery bacillus was alone found.

B. Cases in which the Hiss' Bacillus Y dysentery organism was found.

C. Cases in which the Flexner-Manila bacillus was the only type isolated.

D. Cases in which several members of the dysentery bacillus group were present.

E. Cases in which the variety of the dysentery bacillus present was not determined.

F. Cases in which a lactose-fermenting organism of the "dysentery-like" group was the only pathogenic form isolated.

G. Cases in which pathogenic streptococci were present in addition to some members of the dysentery group.

H. Cases in which streptococci were the only pathogenic organisms found.

I. Cases in which other organisms than dysentery bacilli and streptococci, pathogenic for animals and agglutinating with the patient's blood, were found.

J. Cases with negative special bacteriological findings.

In group A, in which the Shiga type of dysentery bacillus was present to the exclusion of other pathogenic species on aerobic plates, there were four cases. Of these four children three were admitted as instances of malnutrition, to which condition, it was believed, the intestinal infection was added later. The remaining child had high fever on admission. In view of the recent statement of Charlton and Jehle[3] that children infected with the Shiga type of bacillus show more severe and acute symptoms than children infected with the Flexner type, these cases are important.

[3] Charlton and Jehle, *Transactions of the Association of American Physicians*, 1904, xix, 405.

It may be mentioned here that they do not bear out this general statement.

In all these children the bowel derangement began with diarrhœa and mucous stools. In only one child was blood and pus noted. Nausea occurred in two infants. All the cases occurred in July. Two infants recovered, one died, and one on discharge was unimproved. At the autopsy in the fatal case patches of congestion and swelling of the lymphoid structures in the intestine only were found.

The identification of the Shiga bacillus was complete. Further reference to its properties will be made in the more strictly bacteriological part of this paper.

In group B, or the cases in which the type of dysentery organism isolated was the so-called "Y" bacillus, there occurred fourteen cases. Six of these belong to the dyspeptic diarrhœa clinical group, six to the inflammatory ileo-colitides, and the remaining two cases were diagnosed intestinal indigestion. In five children of this series (two with dyspeptic diarrhœa and three with ileo-colitis) marked malnutrition preceded the onset of acute symptoms. Seven of the children were rickety. The stools all showed mucus, and in five cases blood, mucus, and pus were mixed together. The quantity of blood was never large. These patients were often very ill; eight were profoundly toxic and five almost or really collapsed on admission. The temperatures were irregular, often they were subnormal. Vomiting was present in nine cases. Marked abdominal distension occurred twice, and pronounced nervous symptoms four times. The ages varied from one month to thirteen months.

Seven of these children died, five recovered, and two were improved. Four were admitted in June, six in July, and four in August. One autopsy could be made: a well-marked pseudomembranous colitis was found.

These cases were clinically severe types of diarrhœal disease occurring among ill-fed children of the poor. Our experience suggests that this type of dysentery bacillus can cause intense intestinal infections, at least in ill-conditioned infants.

Six infants showed the Flexner-Manila bacillus (Group C.).

The cases were grouped as follows: chronic gastro-intestinal disturbance, one case; mild acute dyspeptic diarrhœa, three cases; malnutrition followed by acute diarrhœa, one case; pseudo-membranous colitis, one case.

Mucus was present in the stools from all the children; blood, as flecks, was noted once only. Fever was not prominent. The children varied in age from two to twenty-one months. Two were admitted in June, two in July, and two in August. Four were discharged and two died. An autopsy in one fatal case showed pseudo-membranous ileo-colitis.

In thirteen infants more than one variety of the dysentery bacillus was isolated. The combinations were as follows [4]:

Groups	I and IV	2 cases.
"	I and II	3 "
"	I, II, and IV	2 "
"	I, II, III, IV, and lactose "B"	1 "
"	II and IV	5 "

These cases pursued as a whole a more severe clinical course than the cases of single infection. In twelve infants the diagnosis of ileo-colitis was made. The remaining infant was marantic and the subject of acute dyspeptic diarrhœa. Tuberculosis was present in two children: once as a miliary infection arising in a caseous bronchial gland, and once as pulmonary phthisis. In the latter case tuberculous ulcers were present in the intestines, besides which, both the Y and Manila type of dysentery bacilli were isolated. In eight children ulceration of the gut was diagnosed. On admission these cases were in fair or poor condition. Four were badly nourished and four were collapsed. They were not especially febrile. The temperatures varied from 97° to 104° F. All the movements contained mucus. In eleven cases pus in considerable quantities was present. The child with tuberculous ulcers passed mucous stools without pus or blood even upon microscopic examination. Pyelitis complicated one case. These infants varied in age from one to thirty months.

One child was admitted in June, five in July, and seven in

[4] The groups arbitrarily indicated by Roman numerals are explained in the bacteriological section of this paper.

August. This seasonal admission is in conformity with the observation that the ulcerative disease tends to appear in the second half of the summer. Nine of the children died, three recovered, and one was improved on discharge. Seven autopsies were obtained. All showed ulcerative ileo-colitis.

In three instances the dysentery bacillus was isolated, but before its type could be determined with accuracy the cultures were lost. Hence these cases will not be described.

The next group comprises two children in which a lactose-fermenting dysentery-like organism was obtained. This organism was first described by Duval and Schorer,[5] and has now been included by Torrey[6] in the pseudo-dysentery group of bacilli. These children showed, one a mild, the other a severe dyspeptic diarrhœa. One child died. No autopsy was obtained.

Eleven cases of combined dysentery bacillus and streptococcus infection were studied. The bacilli consisted of the several types and occurred either singly or combined with one another.

Three of the children showed symptoms of toxic dyspeptic diarrhœa, seven of inflammatory ileo-colitis, and one of intestinal indigestion. One fatal case which came to autopsy showed pseudo-membranous colitis, pyelo-nephritis, and broncho-pneumonia.

The course of the disease in these infants, who ranged from six months or under to thirty months in age, differed materially in severity from infections of the dysentery bacillus alone. The movements always contained mucus, muco-pus was noted in six cases, and more or less blood in six cases. Drowsiness occurred frequently, one child had convulsions at the onset of the symptoms. Three of the children were admitted in June, seven in July, and one in August. Seven children died. Autopsies were made in six cases: two showed the lesions of dyspeptic diarrhœa, three of ulcerative ileo-colitis, and one of membranous colitis. Two children recovered and one improved.

In six cases streptococci only were encountered as the pathogenic bacterial species. In one child only was ileo-colitis noted.

[5] Duval and Schorer, *Studies from the Rockefeller Institute*, 1904, ii, 42.

[6] Torrey, *The Journal of Experimental Medicine*, 1905, vii, 365.

The autopsy in this case showed ulcers in the lower ileum, and hemorrhages and thickening of the large intestine. The five remaining children were examples of malnutrition (two cases), relapsed dyspeptic diarrhœa (one case), acute dyspeptic diarrhœa, (one case), unrecorded (one case).

The stools were mucoid and rarely were pus and blood present. The age varied from three to seven months. Two children were admitted in June, three in July, and one in August. Two children died, one recovered, and three improved. These cases of streptococcal infection were less severe than the cases of combined streptococcal and dysentery bacillus infection.

In the next small group of five cases a pathogenic colon bacillus was isolated twice, a pathogenic bacillus pyocyaneus twice, and a non-pathogenic bacillus pyocyaneus once.

The clinical course of these cases was milder than in the other groups. They were classed with dyspeptic diarrhœa and indigestion. The stools were mucoid and pus and blood were not noted. The children varied in age from three to seventeen months. They had either recovered or improved on discharge.

A final group of ten cases is given in which no pathogenic organism or organism agglutinating with the patient's blood was found. In several of these repeated study of the stools could not be made, and no autopsy was obtained in the fatal cases. In nine cases the clinical diagnosis of toxic infection was made. Once ileo-colitis occurred. Four children were badly nourished; once the symptoms pointed to meningitis. The children varied in age from two to twenty-four months. Eight recovered and two died. The fatal cases were, a suspected meningitis, and an infant which was admitted in a collapsed condition. A detailed account of these cases seems unnecessary.

The study of this series of seventy-four cases of infantile diarrhœa has brought out the fact that forty-two, or 58.3 per cent. of the children yielded, in cultures made from the stools during life or the intestine after death, some variety of the dysentery bacillus, without admixture of streptococci. In eleven additional cases streptococci were associated with dysentery bacilli. Hence dysentery bacilli occurred in 73.1 per cent. of all the cases studied.

If one deduct the two cases in which the lactose-fermenting organism, probably not a true dysentery bacillus, was found, there remain fifty-one cases with dysentery bacilli or 70 per cent. of the whole.

A small number of the children yielded the streptococcus and a still smaller number Bacillus coli communis and Bacillus pyocyaneus as organisms which possibly were directly concerned with the production of the intestinal lesions and symptoms.

The manner of distribution of the several types of dysentery bacilli cannot be determined from this study. It would be unfair and quite without value to base any conclusions on so small a number of cases. But the cases show that the several types of dysentery bacilli now recognized occur most often singly; but that in a fair number of instances a mixture of types exists in the intestine. In considering the frequency of this mixture of types the results of previous studies of the dysentery bacilli as well as our own must be used carefully, for in order to recognize such mixtures the study of large numbers of individual colonies from a given case—a laborious task—is required. Such painstaking studies have been carried out rarely in the past.

If a comparison of the severity of symptoms and fatality be made with the character of dysenteric bacilli infection, it will be found that the most serious lesions and greatest fatality were present among the group of mixed dysenteric infections. Whether this observation is merely accidental, or whether combinations among the types of the bacilli produce more serious consequences than a single type acting alone, cannot be stated without much further study along this line. The observations which we record make this further study desirable.

The characters of the intestinal lesions found at autopsies were extremely various and without definite relation to the types of infecting dysentery bacilli. The lesions might be invisible to the naked eye. They might consist of congestion, or pseudomembrane or ulceration. It would appear that no distinction, except possibly as regards degree, can be drawn between the lesions produced by any of the given types of the dysentery bacillus.

The dysentry bacillus is confined chiefly to the intestinal tract and only rarely penetrates to the mesenteric glands and liver, in which situations it was found in two cases.

The identification of the several types of dysentery bacillus was made by the use of the usual cultural and morphological tests. In the cases studied by us stools containing microscopic or macroscopic pus were the most favorable for the isolation of Bacillus dysenteriæ, this organism being found in 93 per cent. of the cases containing pus. Subdivisions into types were made on the basis of fermentation of sugars and agglutination. The number of the bacilli yielded by the different cases was subject to great variations: in some instances almost pure cultures and in others only a few colonies, after patient search, were obtained.

It will not be necessary to describe in detail the results of the fermentation and agglutination studies. It should, however, be stated that the first were repeated at least three times in each instance. The period of observation was never less than twenty-five days. Two readings were usually made: at the end of five and of twenty-five days.

The cultures which we studied numbered two hundred. These consisted of 172 isolations made by us in the summer of 1904, ten cultures supplied by Dr. Bassett and obtained previously by him from infants, and eighteen cultures from other bacteriologists. The classification into four groups as proposed by Hiss[7] was employed in our studies.

Group I. Original Shiga bacillus group. Monosaccharids are fermented readily; maltose is split slowly and imperfectly. The quantity of acid produced from the first sugars coagulates the serum-water medium, but the amount produced from maltose causes reddening without coagulation. A bacillus agreeing with these group characters was found thirty-five times in fourteen cases, in four cases without admixture of the other types.

Group II. Ferments monosaccharids and mannite early and later ferments saccharose and malteso. This group is represented

[7] Hiss, *The Journal of Medical Research*, 1904, xiii, 151.

by the Y bacillus of Hiss. In our studies we found this bacillus ninety-one times in twenty-five cases, in fourteen of which it was the only type present.

Group III. This group differs very little in its fermentative properties from Group II, the only distinction being its more energetic action upon saccharose. However, in agglutinative properties it also differs from Group II. It is best represented by the Strong culture. We isolated this type of bacillus three times; it was not found independently of other types.

Group IV. The bacilli of this group ferment readily the monosaccharids, mannite, maltose, dextrin, and saccharose. They are represented by the Flexner-Manila bacillus. We isolated this organism forty-four times from sixteen cases. They occurred alone in six cases.

Besides this series of bacilli which are regarded as "true" dysentery bacilli we encountered several times the lactose-splitting bacillus first described by Duval and Schorer [8] in 1904 and later by Duval [9] who regarded it as a new type of dysentery bacillus. Torrey [10] has recently made a careful study of the lactose-fermenting, non-gas-producing dysentery-like bacilli obtained from the diarrhœal stools of children, which he subdivides into three subsidiary types as follows:

Bacillus "A" (Duval and Schorer) ferments the monosaccharids, mannite, and lactose, but not dextrin or saccharose. No member of this group was found in our cases.

Bacillus "B" (Duval and Schorer) ferments the monosaccharids, lactose, mannite, maltrose, and dextrin and slowly ferments lactose. Seven cultures of this organism were obtained from three cases. From two of the cases no "true" dysentery bacilli were obtained.

Bacillus "C" (Duval) ferments the monosaccharids, mannite, lactose, saccharose, malrose, and dextrin readily. No member of this group was obtained from our cases.

In our studies of agglutination we found that the patients' blood in 65.7 per cent. of the cases agglutinated the type of

[8] Loc. cit.

[9] Duval: *Journal of the American Medical Association*, 1904, xliii, 382.

[10] Loc. cit.

Bacillus dysenteriæ isolated from the case, while the blood from the same patients agglutinated the Flexner-Manila type of Bacillus dysenteriæ in only 47.7 per cent. of the cases. Furthermore, the blood in 77.7 per cent. of cases yielding the Flexner-Manila organism agglutinated the same strain of Bacillus dysenteriæ, while in four instances no type of Bacillus dysenteriæ could be isolated though the blood from the cases agglutinated the Manila organism.

In the studies of agglutinations with representatives of the several types of dysentery bacilli and with Bacillus "B" of the lactose-fermenting group the method of Castelanni was employed and the readings were macroscopical. We possessed the following specimens of immune sera: (1) sent by Dr. Shiga from Japan; (2) Flexner-Manila goat serum sent by Dr. Flexner; (3) Y serum sent by Dr. Hiss; (4) rabbit sera prepared by us from "Shiga" and "Strong" and lactose "B" cultures.

We will not give in this place[11] the tables showing the details of our studies upon agglutination, but we will state the general conclusions which we have drawn from them.

The Shiga bacilli (Group I) absorb few agglutinines of the other groups of bacilli, while the Strong bacilli (Group III) partially absorb the agglutinines of the Y bacilli (Group II), and of the Shiga bacilli (Group I).

Absorption with bacilli belonging to Group II reduces the agglutinating value of all sera tested for Group IV and lactose "B," while the agglutinines for Group II in some sera are partially absorbed by Groups I, II, IV, and "B." Group II agglutinines, existing in its own serum, are unaffected by absorption with bacilli of other groups.

Absorption with bacilli of Group III reduces the value of some of the sera for Groups I, II, IV, and "B." The Group III agglutinines are absorbed from some sera by bacilli of Groups II, IV, and "B."

Bacilli of Group IV partially absorb from some sera the agglu-

[11] A full presentation of the data upon which the clinical and bacteriological conclusions given here are based will appear in *The Johns Hopkins Hospital Reports.*

tinines of Groups II, III, and "B." The agglutinines of Group IV are absorbed in all instances by bacilli of Group II and sometimes by those of Groups III and "B."

Lactose bacilli absorb the agglutinines of Groups II, III, and IV, while the agglutinines of "B" are absorbed by bacilli of Groups II, III, and IV.

The absorption of agglutinines by the different representatives of the groups of dysentery bacilli varies with the type of organism used in immunization. It is not improbable that a further variation will be found to depend upon the animal used for immunization. Our studies do not establish strong and definite specificity in agglutination among the several group-types, as the differences noted were usually those of degree only. Apparently the Flexner-Manila bacillus yields on immunization the greatest amount of common agglutinines since its serum is active against all the five group-types of bacilli studied by us.

STUDIES UPON THE INFLUENCE OF TENSION IN THE DEGENERATION OF ELASTIC FIBRES OF BURIED AORTÆ.

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Plates XVII, XVIII, and XIX.

The experiments described in this paper were carried out with the object of studying the power of resistance possessed by elastic tissue to degenerative changes, the order of such changes as do occur when this resistance is overcome, and the effect of continuously applied tension upon these fibres.

Space does not permit of more than a brief review of the investigations already made by pathologists upon this tissue under various abnormal conditions. For more detail the reader is referred to a very concise paper by Jores, in Lubarsch and Ostertag's *Ergebnisse der allgemeinen Pathologie* (1902, Jahrgang VIII, p. 590).

Disappearance of Elastic Fibres.—Under various pathological conditions, organs, which show in health a large quantity of elastic tissue, exhibit a very marked decrease, if not total absence, of these fibres. The cause of this disappearance is much disputed. Du Mesnil de Rochemont considers that degeneration of elastic fibres is due to chemical action. He comes to this conclusion from observations made upon tuberculous material. He believes that the breaking down and disappearance of the elastic tissue is the result of free toxins which are the products of the bacillus of tuberculosis. In experimental pulmonary tuberculosis in animals produced by injecting cultures of the bacillus into the vascular system, Wechsberg noted a disappearance of the elastic fibres of the lung; this condition was recognizable within six hours and was very marked at the end of two days. He attributed it to the action of toxins of the micro-organism.

In sections of lupoid material, du Mesnil de Rochemont found a narrow marginal zone, about a line in width, which was free from

these fibres. Here again he thinks that their absence is due to the action of toxins. Miller has observed the disappearance of this tissue in pulmonary gangrene and in syphilitic lungs within the areas of miliary gummata.

Obermüller, studying the changes in the vaginal wall in a case of prolapsus vaginæ, found an entire absence of elastic fibres in that part of the mucous membrane which was prolapsed and therefore exposed to mechanical irritation. This he thought was the result of fibrosis, secondary to the continual friction. The same author finds a similar absence of elastic fibres in those portions of the vaginal mucous membrane which have been subjected to prolonged pressure from constant wearing of a pessary. I can bear out Obermüller's observations concerning the disappearance of elastic fibres with prolapsus vaginæ. I have had the opportunity of examining sections from a case of prolapse of six years' duration. There was an almost entire absence of these fibres in the tunica propria and an extensive increase of white fibrous tissue. An obliterating endarteritis was also present.

Melnikow-Raswedenkow found, in testicular tuberculosis, a marked degeneration of the elastic fibres about the inflammatory zone of the tubercles, but in the necrotic centres he found the fibres intact. The explanation he advanced was, that the factor in the production of degeneration was the inflammatory cell infiltration and not the presence of toxin, since if the latter were the cause, the fibres would not remain in the necrotic tissue, which, one would suppose, would be surcharged with toxins. Schmaus finds that elastic fibres disappear very much more readily in the presence of granulation tissue than in true tubercles.

Jores agrees with the explanation offered by Melnikow-Raswedenkow. He quotes the work of his pupil; elastic tissue was exposed to inflammatory exudates and transudates at blood-heat in an incubator and was studied at various intervals, but in no case were changes found which at all resembled those occurring in inflammatory conditions during life though the tissue was in close contact with toxins which were present in the exudates and transudates.

In acute conditions a number of observers have found a

disappearance of the elastic elements. Others however deny that they are absent and claim that the disappearance is only apparent, elastic tissue being invisible as the result of loss of staining power. Those who hold the latter view are Unna, who notes this condition in œdema, Luithlen, Passarge, Jores, and others. Jores believes that this change explains the apparent loss of elastic tissue in pulmonary stasis, œdema, and lobular pneumonia.

Fragmentation.—With lesions such as aneurysm, arterio-fibrosis, etc., the elastic fibres are found broken into fragments and the condition may be designated as fragmentation. Koester believes that this is of inflammatory origin and considers the small fibrotic areas which are found in vessel-walls—the so-called Kœster's spots—the result of a similar process. In these areas the elastic fibres are segmented, but this he claims is primarily due to the inflammation. Fabris supports him in this explanation; he produced necrosis experimentally, and then investigated the change which resulted in the elastic fibres. He found extensive fragmentation of this tissue. Jores appears to hold the same view concerning the condition. He does not consider, however, the gaps, which Thoma, Manchot, and others have described as isolated tears, to be anything other than the physiological openings or fenestra of the laminæ elasticæ.

Among those opposing this view are von Recklinghausen, Manchot, and Dmitrijeff. These authors hold that mechanical action is the cause of this breaking. Manchot observed in the walls of aneurysmal blood-vessels fragmentation of the elastic fibres, but no indications of inflammatory reaction in the other tissues of the wall. He considers it very unlikely, therefore, that the elastic tissue is attacked by inflammation while other tissues escape. The same observer has found interruptions in the course of individual fibres, and these, he thinks, are the result of excessive tension. Thoma advances the same theory and apparently believes that these isolated tears are the initial stage of diffuse arterio-fibrosis, while the patchy variety commences in foci where several fibres are involved.

Dmitrijeff thinks Koester's spots are small areas of granulation tissue occurring around isolated tears and that they are the result

of irritation caused by these breaks. Katsurada experimented by pinching the skin of animals, with forceps, and then examined the site of injury at different periods. He found that fragmentation did not immediately follow the injury, but occurred when the growth of fibrous tissue, consequent to stimulation caused by the trauma, became excessive.

Granular Degeneration.—With one form of degeneration the elastic fibre is represented by a line of irregular granules which take the elective elastic stains. Weiszmann, Neumann, and Manchot observed this form of degeneration in the early stages of arterio-fibrosis and aneurysm. Dmitrijeff found the same condition and obtained an elacin reaction in some of the granules. Miller has noticed this condition in the tubercles occurring with acute miliary tuberculosis. With syphilitic involvement of the vascular system, Abramow has observed extensive granular disintegration of the elastic fibres.

Jores has described a fatty degeneration of elastic fibres occurring in the absence of other signs of a degenerative process. In association with this condition he has noticed granulation of neighboring fibres and for this reason believes that both these changes are due to the same cause.

Calcification.—The elastic fibres may be impregnated by a lime salt; calcification occurs in cases of sclerosis involving the walls of blood-vessels or other tissues containing elastic fibres. Kockel has described incrustation of elastic fibres in calcified lung tissue. The deposit, he found, consisted of fine granules laid down along the fibres which were frequently broken. These granules stained well with resorcin-fuchsin. Von Davidsohn made a similar observation and likens the appearance presented by the fibre under these conditions to a chain of anthrax bacilli. Fragmentation in this tissue he considers analogous to fragmentatio myocardii. He does not believe that calcification begins in the fragmented fibres.

In some pathological conditions of the thyroid gland, Jores found calcareous degeneration of the elastic elements. The deposit was strictly limited to the elastic lamina. Matusewiez and Rona describe similar changes. The last-named writer found some of these granules occurring as inclusions in giant cells.

Upon removing the calcium salt from them he obtained a good elastic stain with orcëin and therefore was led to believe that the giant cells were responsible for the presence of calcium. In addition to the calcium salt, Rona found traces of iron. Gierke had shown, previous to Rona's observation, that iron is usually present with this form of degeneration.

Methods.—In order to study degenerative changes in normal elastic tissue an aorta obtained under aseptic conditions from the body of a healthy guinea-pig killed at the time required was buried within the tissues of another animal of the same species. The hair of this animal was removed over the site of operation by means of a depilatory powder.[1] Chloroform was administered to animals to be killed, but when recovery from the anæsthetic was desired it was found safer to employ a mixture of alcohol, chloroform, and ether (one, two, and six parts respectively).

The incision into the second pig extended through the skin, subcutaneous tissues, and the more superficial muscular tunic of the abdominal wall. With the handle of a scalpel a small pouch or pocket was formed by separating the muscular layers and into this pocket was placed the tissue to be buried. In order to prevent it from curling up, two anchor-ligatures of catgut were attached to the ends of the vessel; these ligatures were then led through the outer wall of the pouch, one at each end, and when drawn fairly taut were sealed to the skin. The wound was closed by catgut sutures and dressed with sterilized cotton and collodion.

In some instances (Groups B and C) in which it was desired to place the elastic elements under tension, the aortæ were distended under pressure with a sterile three per cent. solution of agar-agar in 0.85 per cent. sodium chloride, in other instances with sterile paraffin melting at 43° C.; the vessels were then doubly ligated at each end and excised.

The animals in which the collapsed vessels were buried were killed at the end of 3, 6, 12, 27, 33, 42, 54, 75, 106, 150, and 303 days, while those containing the distended aortæ, at the end of 10, 12, 15, 21, 27, 30, 35, and 50 days. After death the sections

[1] A very good formula for this powder will be found in Crocker's work on Diseases of the Skin, in the section on Hypertrichosis.

of abdominal wall containing the buried structures were removed, fixed and hardened in formalin (10 per cent.) solution and alcohol, and imbedded in paraffin.

The stains employed were hæmatoxylin and eosin, Van Gieson's fluid, resorcin-fuchsin, Weigert's elastic stain, orcëin, Unna-Taenzer's elastic stain, Wasserblau-safranin and three per cent. silver nitrate solution, and Schmorl's modification of v. Kossa's calcium reaction. Special tests for the presence of phosphates and chlorides were made.

GROUP A.—AORTÆ BURIED IN UNDISTENDED CONDITION.

EXPERIMENT 1.—*Aorta after three days.* The vessel lies loosely imbedded in a quantity of granulation tissue, in the intermuscular space of the abdominal wall. The elements of the muscle and connective tissue of the buried aorta exhibit somewhat impaired reactions to the stains employed. The elastic structure of the vessel-wall stains well with the elective elastic tissue methods and exhibits no indication of a degenerative change. The lumen of the artery remains patent.

EXPERIMENT 2.—*Aorta after six days.* The buried vessel is firmly imbedded in recent granulation tissue. The lumen is occluded by a coagulum, in which commencing organization may be seen. There is a decided loss in quantity as well as in staining power of the muscle and connective tissues of the vessel but the elastic fibres are as yet apparently unchanged.

EXPERIMENT 3.—*Aorta after twelve days.* At this period the muscle and connective tissues have apparently been entirely absorbed, and the only remains of the buried vessel are the elastic framework. This structure is normal in appearance and to the elective stains shows a good reaction. The elastic fibrils, at the periphery of the vessel, exhibit a slight tendency to fragment, but this condition is strictly limited to fibrillar portions of the elastic structure.

EXPERIMENT 4.—*Aorta after twenty-seven days.* As in the previous specimen, the elastic tissue alone remains. A well-organized clot occupies the site of the occluded lumen. Though the elastic fibres generally take the specific stains well, yet there are small portions which indicate, by an impaired tingibility, an alteration of some kind. These areas occur chiefly among the peripheral and intimal fibres, the medial ones having so far escaped. In places also is seen a fairly well-marked fibrillation. No reaction is obtained upon using a special stain for degenerated elastin—the so-called elacin of Unna.

EXPERIMENT 5.—*Aorta after thirty-three days.* The aorta bears a close resemblance to the vessel buried twenty-seven days. In this specimen, however, the elacin reaction is obtainable, though it is not very marked. It appears to be confined to the internal elastic lamina. The newly forming connective tissue shows a tendency rather to compress than to invade the elastic structure. Fragmentation also occurs.

EXPERIMENT 6.—*Aorta after forty-two days.* An alteration of the staining

powers of the elastic tissue in this specimen is very conspicuous. Both to resorcin-fuchsin and to acid orcëin these fibres exhibit a marked resistance, but when Wasserblau-safranin is employed they show extensive elacin degeneration. The internal elastic lamina shows several breaks, as do some of the other heavy fibres. Some of the fibres are fibrillating and the fibrils formed by this process of splitting-off are, in addition, undergoing granular disintegration. No reaction is obtained with Schmorl's calcium test. (Plate XVII, Fig. 5 and 6.)

EXPERIMENT 7.—*Aorta after fifty-four days.* There is a more marked loss of staining power with the elective stains than in the earlier specimens. Fibrilation, with its accompanying granular disintegration, and condensation of the elastic fibres continue. The elacin reaction is marked, but Schmorl's test for calcium yields only negative results.

EXPERIMENT 8.—*Aorta after seventy-five days.* Extensive changes in the elastic fibres are discernible. With both resorcin-fuchsin and acid orcëin there is a marked tendency to stain excessively and diffusely. The elacin reaction is present but not more marked than in the aorta after fifty-four days. With Schmorl's nitrate of silver test for calcium small dark areas are to be seen here and there in the course of some of the heavier fibres, including the lamina elastica interna. As the reaction is not marked there might be some doubt in stating that it is present at this date.

EXPERIMENT 9.—*Aorta after one hundred and six days.* There is marked hyper-tingibility of the elastic structure upon using the elective stains and there is the same heavy and diffuse reaction that is seen in the aorta after seventy-five days. With Wasserblau-safranin the reaction, though present, is less marked than in the previous specimen. Upon the application of Schmorl's test, however, there is seen an extensive calcareous deposition in the paths of the elastic fibres, especially of the lamina elastica interna. This deposition appears to be strictly limited to the fibres themselves—no deposit being seen except where granular disintegration has been marked. The sections cut with difficulty. (Plate XVIII, Fig. 7, 8, and 9.)

EXPERIMENT 10.—*Aorta after one hundred and fifty days.* The elastic structure is markedly condensed. The fibres show a hyper-tingibility similar to that found in the vessel buried one hundred and six days. The elacin reaction is absent, but Schmorl's test reveals a very complete impregnation of most of the elastic tissue by a calcium salt. Upon comparing one section of this aorta stained for calcium, with another stained with resorcin-fuchsin or with acid orcëin, the extent of this impregnation is better appreciated. Difficulty was encountered in cutting sections of this vessel also. (Plate XVIII, Fig. 10 and 11, Plate XIX, Fig. 12.)

EXPERIMENT 11.—*Aorta after three hundred and three days.* This specimen exhibits a large portion of the elastic structure of the buried vessel. The remains are, however, extensively broken and are calcified, but the extent of calcification is not so marked as in the specimen of one hundred and fifty days The elective elastic stains are taken excessively and diffusely. No reaction for elacin is obtained. (Plate XIX, Fig. 13, 14, and 15.)

GROUP B.—AORTÆ DISTENDED WITH AGAR-AGAR AND BURIED.

EXPERIMENT 12.—*Aorta after twelve days.* The aorta is found well imbedded in granulation tissue. The distending medium, agar-agar, is broken into several parts by invading bands of newly forming tissue. The muscle and connective tissues have apparently been absorbed, the elastic structure of the vessel wall alone remaining. As a result of the tension placed upon the vessel during the period of experimentation, there is a marked diminution in the number of fibrils and smaller fibres present. A fibrillation of some of the larger fibres occurs, and fragmentation, though not marked, is present. The reaction to resorcin-fuchsin and to acid orcëin is apparently unaltered.

EXPERIMENT 13.—*Aorta after fifteen days; infected.* The effect of the continuous pressure is well shown in this specimen. Fragmentation, fibrillation and granular disintegration are present, the first not marked, the second and third more conspicuous. Very active cell invasion of the buried part is seen, and this no doubt accounts for the decrease in the peripheral fibrils. The tissue reacts well to the elective stains and no indications of elacin are found. In certain portions of the section the lamina elastica interna is found to be stripped from the subjacent structure and bulges into the lumen. This condition is caused by a collection of inflammatory cells beneath this membrane. In some places such as these the elastic fibres present an eroded appearance. In this specimen an additional factor has been infection. (Plate XVII, Fig. 1 and 2.)

EXPERIMENT 14.—*Aorta after twenty-one days.* A condition very similar to that described above is present. A decrease in the peripheral fibres and fibrillation are marked. The elective stains are well taken and there is no evidence of the presence of elacin. The fibres are not stretched to the same extent as in the other experiments. The distending medium is being rapidly absorbed, the lamina elastica interna shows numerous breaks and in places exhibits signs of erosion. Fragmentation though not marked occurs in other parts of the section.

EXPERIMENT 15—*Aorta after thirty days.* A marked decrease in the quantity of elastic tissue is at once noticed. Fragmentation is marked and the fibres are split and in some places are represented by granular masses. The internal elastic membrane is extensively broken and shows plainly indications of erosion. Only small portions of the agar-agar remain unabsorbed. The whole structure exhibits hyper-tingibility, but when treated with Wasserblau-safranin yields no indications of elacin. There is a marked tendency of the newly forming tissue to compress or condense rather than to invade the elastic structure. (Plate XVII, Fig. 3 and 4.)

GROUP C.—AORTÆ DISTENDED WITH PARAFFIN AND BURIED.

EXPERIMENT 16.—*Aorta after ten days.* The vessel is imbedded in a quantity of new fibrous tissue. The muscle and connective tissues of the buried vessel cannot be identified. The elastic fibres are well shown, however, by the elective stains. There is a marked absence of fibrils and a slight breaking of the outermost fibres.

EXPERIMENT 17.—*Aorta after twenty-seven days.* The elastic fibres alone

remain to represent the vessel buried in this experiment. These show marked fibrillation and granular degeneration, and the elastic structure as a whole, shows a decrease in volume. A reaction to the specific dyes is obtained, though there is no indication of elacin. In this experiment only a moderate amount of tension was exerted.

EXPERIMENT 18.—*Aorta after thirty-five days.* In this experiment a great deal of tension was exerted upon the vessel wall. Upon sectioning and staining, there is found to remain only a very small quantity of elastic tissue. The remains are fibrillated and granular and take the elective stains fairly well but exhibit no signs of elacin.

EXPERIMENT 19.—*Aorta after fifty days.* The degree of tension exerted about equalled that in the preceding experiment. There is absolutely no trace of elastic tissue to be found.

In studying the impregnation of elastic fibres by certain salts, it was found, as the result of the reactions given below, that calcium phosphate, principally, but also a small quantity of chloride were present. Klotz, by the use of Sudan III, demonstrated the formation of soaps preceding calcareous deposition. This test, however, was not applied to the tissues in my experiments.

For the demonstration of calcium, sections of the tissue were treated with a freshly made solution of hæmatoxylin (one fourth per cent.) in distilled water: while for phosphates and chlorides the reactions described by Professor A. B. Macallum and Schmorl's modification of von Kossa's test were used. In separation of chlorides from phosphates, the reagent used was that of Schmorl (three per cent. silver nitrate in distilled water plus one and a half per cent. nitric acid). Since silver phosphate is soluble in dilute nitric acid while the chloride is not, any subsequent darkening upon exposure of the sections to light indicated the presence of silver chloride.

From the study of the specimens described above, the following conclusions are, I believe warrantable:

1. Elastic tissue offers a very marked resistance to degenerative processes; this is especially noticeable when the changes which it undergoes are compared with those occurring in other tissues under similar conditions.

2. The power of resistance is lowered, apparently, when this tissue is placed under tension.

3. Degeneration of elastic fibres, when not influenced by ten-

sion, is indicated by the following changes which occur in the order given: (a) fibrillation or splitting of the fibres, (b) loss of tingibility, (c) fragmentation and granulation; then occurs either (d) absorption or (e) formation of elacin from elastin, followed by (f) a tendency to over and diffuse staining with the elective stains, and finally (g) impregnation of the elastic fibres with a calcium salt.

4. Under tension, elastic fibres may be absorbed without visible change except progressive thinning, or may fibrillate, undergo granular disintegration, and then be gradually absorbed.

5. Under tension and under the other conditions of these experiments, elastic fibres, apparently show no tendency to undergo impregnation by calcium salts.

6. The elective elastic stains are not true indicators of the condition of elastic fibres in certain stages of degeneration, unless used in conjunction with tests for elacin (Unna's method) and calcium (Schmorl's method).

In conclusion I wish to express my thanks to Professor J. J. MacKenzie at whose suggestion this work was undertaken and who has assisted me with his advice throughout its prosecution.

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EXPLANATION OF PLATES.

Plate XVII.

Fig. 1.—Low magnification of aorta buried fifteen days. Experiment 13. The elastic fibres are well stained. To the right, the lamina elastica interna is separated from the subjacent elastic fibres by a collection of inflammatory cells. To the left, fragmentation of the elastic fibres is observed. (Resorcin-fuchsin stain.)

Fig. 2.—High magnification of specimen shown in Fig. 1. In addition to the stripping off of the lamina elastica interna, the segmented condition of the fibres is well shown. (Resorcin-fuchsin stain.)

Fig. 3.—Low magnification of section of aorta buried thirty days. Experiment 15. The lower portion of this figure exhibits marked thinning of the elastic structure. The whole specimen shows extensive breaking of the elastic tissue, particularly noticeable to the left. (Resorcin-fuchsin stain.)

Fig. 4.—High magnification of specimen shown in Fig. 3. Segmentation of the fibres is shown. The elastic tissue stains well, but exhibits marked condensation. (Resorcin-fuchsin stain.)

Fig. 5.—Low magnification of an aorta buried forty-two days. Experiment 6. This photomicrograph shows well the impairment of tingibility of the elastic element—as seen in the lamina elastica interna and in some of the coarser fibres. Fragmentation is also shown. (Resorcin-fuchsin stain.)

Fig. 6.—High magnification of the specimen shown in Fig. 5. The inequality of staining of the elastic fibres in this specimen is very plainly brought out. (Resorcin-fuchsin stain.)

Plate XVIII.

Fig. 7.—Low magnification of aorta buried one hundred and six days. Experiment 9. A marked hyper-tingibility of the remains of the elastic structure is present; the stain is taken deeply and diffusely. The elastic fibres show extensive segmentation. (Resorcin-fuchsin stain.)

Fig. 8.—Low magnification of the aorta shown in Fig. 7. (Schmorl's reaction for calcium.) Calcification of the fibres is very plainly demonstrated. The extent of the condition will be better appreciated if this figure be compared with the preceding one.

Fig. 9.—High magnification of same specimen as Fig. 7. (Resorcin-fuchsin stain.)

Fig. 10.—Low magnification of section of an aorta buried one hundred and fifty days. Experiment 10. This specimen exhibits hyper-tingibility which is very much more marked than that occurring in the aorta buried one hundred and six days. (Resorcin-fuchsin stain.)

Fg. 1.

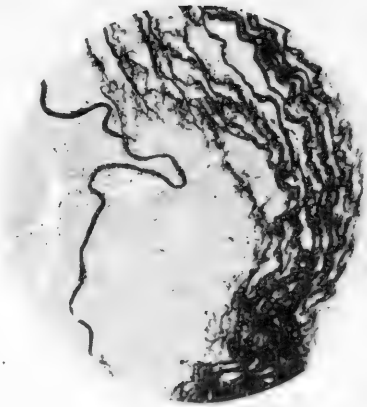

Fig. 2.

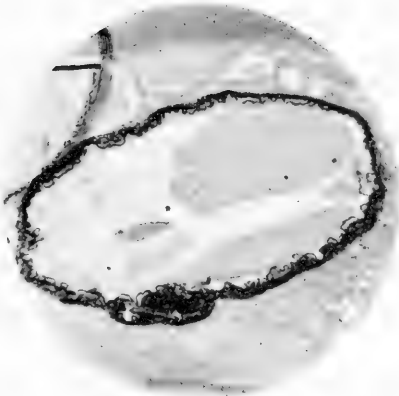

Fig. 3.

Fig. 4.

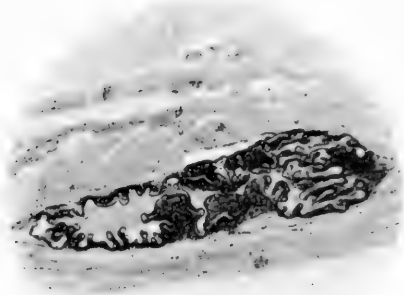

Fig. 5.

Fig. 6.

Fig. 7.

Fig. 8.

Fig. 9.

Fig. 10.

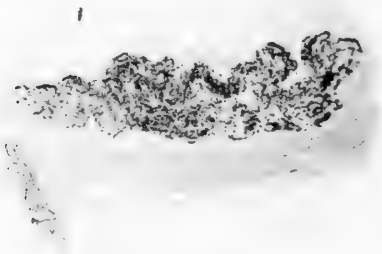

Fig. 11.

Fig. 12.

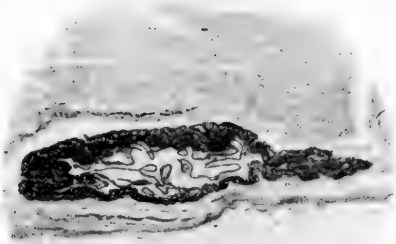

Fig. 13.

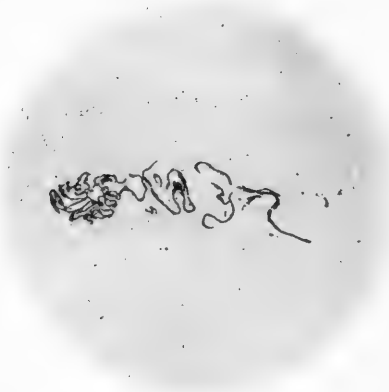

Fig. 14.

Fig. 15.

Fig 11.—Low magnification of the aorta shown in Fig. 10. (Schmorl's reaction for calcium.) Impregnation of the fibres with calcium is very marked and results in an almost complete cast of the original elastic framework.

PLATE XIX.

Fig. 12.—High magnification of same specimen as Fig. 10. (Resorcin-fuchsin stain.)

Fig. 13.—Low magnification of an aorta buried three hundred and three days. Experiment 11. The fibres in this specimen exhibit a staining reaction which corresponds to that shown in Fig. 7. and 10. (Resorcin-fuchsin stain.)

Fig. 14.—Low magnification of same specimen as Fig. 13. (Schmorl's calcium reaction). The extent of calcification of the elastic fibres is shown. It is not nearly so extensive as that occurring in the aortas buried one hundred and six and one hundred and fifty days.

Fig. 15.—High magnification of same specimen as Fig. 13. (Resorcin-fuchsin stain.)

EXPERIMENTAL MYOCARDITIS; A STUDY OF THE HISTOLOGICAL CHANGES FOLLOWING INTRAVENOUS INJECTIONS OF ADRENALIN.*

By RICHARD M. PEARCE, M. D.

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PLATE XX.

During a recent investigation[1] by Dr. Stanton and myself of the lesions occurring in the aorta of the rabbit as the result of repeated injections of adrenalin, the heart muscle, in the few instances in which it was examined, was seen not infrequently to be the seat of very definite degenerative lesions and occasionally also of proliferative changes. The association of such conditions with an experimental vascular lesion suggested the possibility of producing experimentally a chronic myocarditis analogous to that seen not infrequently in man in connection with grave arterial disease. As the material at hand was insufficient for a comprehensive study a second series of investigations was inaugurated. In these an attempt has been made to study the sequence of the changes and to determine to what extent they are due to the direct action of adrenalin and to what extent secondary to the arterial lesions. Few of the investigators who have produced vascular lesions with adrenalin have described the associated myocardial changes. Fischer[2] mentions the occurrence of necrotic and fibrous changes and, rarely, calcification. K. Ziegler,[3] in a series of eight animals, found changes very similar to those hereinafter detailed.

Methods.—These were practically the same as in the earlier experiments by Dr. Stanton and myself, except that the initial dose was placed at one-tenth cubic centimetre, and rapidly raised to five-tenths and occasionally higher. All injections

* This investigation was conducted under a grant from the Rockefeller Institute for Medical Research. Read before the Johns Hopkins Medical Society, December 18, 1905.

were made into the ear vein, and usually on alternate days. As previous experiments had shown that ten to twelve injections of adrenalin were sufficient to produce well marked arterial lesions, the injections in this series with a few exceptions were thus limited and the animals were then allowed to remain without further treatment for periods varying from one to three weeks. Thus the animals which survived the treatment represent an average injection period of twenty-four days, and a total elapse of from twenty-seven to forty-five days, a sufficient time, as indicated by the few preliminary observations, for the development of proliferative lesions in the myocardium. The animals dying during the course of the injections furnished sufficient material for the study of early degenerative changes.

The histological methods were limited to the use of Scharlach R for frozen sections, and of hæmatoxylin and eosin and Mallory's connective tissue stain for material hardened in Zenker's fluid and imbedded in paraffin.

Results.—Thirty-six rabbits were injected. These may be divided into two groups: the first group including fourteen animals which died during the period of injection, and the second group, twenty-two animals, which received from seven to fifteen injections and were killed at various intervals thereafter. The first group represents animals receiving from one to five injections, of which none died later than the tenth day. All animals dying in this stage succumb apparently to circulatory and respiratory disturbance and present at autopsy acute dilatation of the heart, œdema and congestion of the lungs, and occasionally hæmorrhages from the serous and mucous membranes or in the interior of organs. No macroscopic lesions of the aorta are demonstrable. When death follows the injection within a few minutes the cardial dilatation, especially after the fourth or fifth injection, is extreme. Under such circumstances the heart loses much of its conical shape; the apex is more or less rounded and the overdistended auricles project laterally and greatly broaden the base. The left ventricle is very anæmic and its pale almost bloodless substance is in sharp contrast to the blue-black color of the thin walled auricles.

Degenerative Changes.—The heart muscle of two of three rabbits dying within a few minutes after a single injection shows a well marked œdema causing more or less separation of the muscle fibres: in the third animal the œdema is slight. In all three the muscle fibres show irregular swellings, and the striations are somewhat indistinct. In one, multiple fractures of more or less homogeneous fibres are frequently seen, but as these conditions could not be found in either of the other hearts, they were considered as probably artifacts.

But one animal died after two injections. This death occurred on the fourth day, thirty-six hours after the second injection. The œdema of the myocardium is so great as to cause wide separation of the muscle fibres in many areas. In some of these areas the fibres have lost their striations and are very granular. A few small areas of intense œdema take a diffuse blue stain similar to myxomatous tissue.

Three animals died after three injections. The notes on one of these follow:

RABBIT No. 25.—Male, weight 1670 grammes. The initial dose was one-tenth cubic centimetre of a one to one thousand solution of adrenalin injected into the ear vein. On the following day the dose was raised to two-tenths and after an interval of two days it was repeated. The animal was found dead seventeen hours after the last injection. At autopsy œdema and congestion of the lungs and extreme dilatation of the heart are present. Histologically the heart reveals focal areas of most intense œdema. These occur not only in the wall of the ventricle but also in the papillary muscles. Crossing these areas are strands of connective tissue in the midst of which fragments of heart muscle may occasionally be seen. Some of these fragments stain well while others are merely shadows of fibres in the midst of the finely granular coagulated serum. Some have ragged irregular ends pointing definitely to displacement by fracture. Elsewhere the fibres are widely separated by a serous fluid, are very granular, have swollen nuclei, stain lightly with eosin, and have but indistinct striations. On cross section many of the fibres show a clear zone about the nucleus, evidently a perinuclear œdema. Occasionally, bundles of fibres appear as if torn across, the space between being filled with serum. The branching of the fibres is brought out well by the œdema. Swollen hyaline fibres are not infrequent. The larger blood vessels are widely distended; the capillaries on the other hand are not prominent. No lesions are seen in the walls of the blood vessels.

In a second animal of this period which received three injections of one-tenth cubic centimetre the œdema was not so prominent, but on the other hand more extensive granular and hyaline

degeneration of the fibres was present. After the fourth injection one animal died and after the fifth, six. These last represent the period of greatest mortality as well as the gravest degenerative lesions. All animals dying at this time showed extreme dilatation of the heart and histologically, in addition to the changes just described, a severe granular and hyaline transformation with more or less necrosis and disorganization. This condition is illustrated by the following protocol:

RABBIT No. 6.—Male, weight 1720 grammes. On September 26, 29, and 30 one-tenth cubic centimetre of adrenalin solution was injected into the ear vein; on October 2 and 3, two tenths. The animal died during the night of October 3. Upon post-mortem examination the heart was found to be dilated, the left ventricle pale and bloodless, and the lungs œdematous; the other organs were normal. Sections from three levels of the heart show general œdema and extreme disintegration of the muscle fibres. On both cross and longitudinal section the latter are seen to be irregularly swollen, granular, without striation, and not infrequently hyaline. The nuclei are swollen and frequently surrounded by a zone of œdema. In many fibres with extensive vacuolization of the protoplasm the nucleus does not stain. About such fibres small accumulations of mononuclear leucocytes are occasionally present and in some instances foci of epithelioid cells. In areas of extreme degeneration the outlines of the muscle fibres on cross section are indistinct and the picture is that of a uniformly granular mass with regularly placed lightly staining chromatic rings. These various changes affect the papillary muscles also. No changes in the vessels of the heart are demonstrable. Histological examination of the aorta is negative.

In other animals of this period the degenerative changes in the heart were of the same general character though varying more or less in degree. In two, a slight diffuse growth of connective tissue between the muscle fibres occurred.

Attempts to demonstrate fat in frozen sections of formalin hardened material treated with Scharlach R and counterstained by hæmatoxylin were uniformly negative. This method however revealed, in a few hearts, isolated fibres or small groups of fibres taking a diffuse yellow stain. These were more or less hyaline, and without nuclei; they however occurred in close relation to other similar fibres which did not thus react. This reaction has not as far as I am aware been previously described and its significance is not apparent.

The lesions in these hearts are due, manifestly, either to the

direct toxic action of adrenalin upon the heart-muscle or to mechanical disturbances of the circulation caused by the effect of adrenalin on the vascular mechanism. They cannot be secondary to alterations in the walls of the large vessels, for in this series none of the animals exhibited macroscopic lesions of the aorta and in but three were degenerative changes evident microscopically.

Proliferative Changes.—Of the twenty-two animals survivng seven to fifteen injections, all but three showed degenerative changes in the myocardium, though seldom of the severe grade observed in the above descriptions. In twelve, more or less definite new connective tissue formation was evident. This new tissue was present, in some hearts, as focal accumulations of fine connective tissue about individual hyaline or granular fibres or about small groups of such fibres. In others a diffuse arrangement of new tissue was demonstrable in considerable areas of the myocardium and in two cases a very severe and diffuse fibrous myocarditis existed. The new tissue as a rule was most abundant about the blood vessels but had no relation to the sub-epicardial tissue or to the endocardium. It was very prominent in the papillary muscles. All parts of the ventricle suffered, though it any difference existed, the lesions were more severe at the apex. Focal accumulations of leucocytes were not prominent in the more chronic lesions, though occasionally present in the earlier conditions. When present these were usually of the lymphoid cell type. No suppuration or other evidence of infection was seen at any time; the lesion apparently was a pure connective tissue proliferation following degeneration of muscle fibres. The following protocol is that of the most advanced lesion observed:

RABBIT No. 22.—Male, weight 1685 grammes. During a period of eleven days seven injections of adrenalin varying in amount from one-tenth to eight-tenths cubic centimetre were given (total amount five and two-tenths cubic centimetres). The animal was killed by chloroform five days after the last injection.

Autopsy.—Weight 1650 grammes. A small amount of clear serous fluid is found in the peritoneal and pleural cavities. The heart is large and firm; the walls of the left ventricle are distinctly thickened; the auricles are dilated. The entire circumference of the aorta at its origin is the seat of closely massed, depressed, round or oval, calcified areas which form a complete ring about the

vessel and extend irregularly into the sinuses of Valsalva and along the ascending portion of the aorta for a distance of one and two-tenths centimetres. The orifices of the coronary arteries, in the natural position of the aorta, are entirely obscured but by pressure on the heart substance their presence is indicated by the escape of minute points of blood. These lesions cause considerable deformity of the vessel. A few isolated plaques of similar appearance are scattered elsewhere in the aorta and a considerable number are grouped about the origin of the cœliac axis.

Histology.—Sections through the entire ventricle from base to apex show extreme degeneration of muscle fibres and a new formation of connective tissue diffusely arranged, for the most part, but with here and there focal areas in which the muscle fibres are entirely absent. The muscle fibres as a rule are swollen, vacuolated, and exceedingly granular, though some are atrophied. Fibres with irregular constriction and swelling and more or less hyaline transformation are not uncommon. The nuclei are distorted, stain poorly, and frequently are entirely absent. In some fibres the nucleus is contracted and surrounded by a clear zone; in other fibres the nucleus is greatly swollen. The connective tissue is more abundant in the mid and inner portions of the ventricle and but slight beneath the pericardium and in the papillary muscles. The focal areas are more numerous at the apex of the ventricle and towards the endocardial surface. The connective tissue is very loose and œdematous Between the elongated wavy vesicular nuclei is considerable intercellular substance practically free of lymphoid cells. While the arrangement of the new tissue is in general diffuse it is frequently more abundant about the blood vessels. In the focal areas the fibrillated character of the new tissue is very evident. The walls of the blood vessels show no change. Sections stained by Mallory's connective tissue method show everywhere between the muscle fibres a more or less well marked blue reticulum, while the focal areas take a deep stain relieved only by the deep red of fragments of hyaline fibres. The methods for demonstrating elastic tissue show a diffuse increase of fine elastic fibrils in the denser areas of connective tissue; they reveal no changes in the blood vessels.

Control Observations.—Although the literature treating of various experimental lesions of the myocardium of the rabbit contains no references to the occurrence of degenerative and proliferative changes in the hearts of apparently normal rabbits, it seemed advisable to determine definitely this point. As the sacrifice of a series of normal rabbits appeared undesirable the sections of heart muscle from a large number of animals injected with various organs and fluids of the dog during the past few years, in the course of several investigations of cytolysins, were utilized. A careful study of this material failed to reveal fibrous changes and showed few degenerative lesions. The latter were of slight

degree and never of the type occurring after the administration of adrenalin.

Mode of Action of Adrenalin.—The association of a severe fibrous myocarditis with vascular lesions involving the orifices of the coronary arteries, as in the experiment given above in detail, suggests that the latter condition must have some causal relation to the myocardial changes. Indeed in the early part of the investigation it was thought that such a relation would eventually be demonstrated. Such, however, does not appear to be the case, for in one instance a myocarditis of equally severe type developed in the entire absence of lesions in the aorta. Moreover in six of the twelve animals exhibiting changes in the myocardium no lesions of the aorta were present, and in three of the remainder the lesions were but slight. The fibrous changes in the myocardium would appear therefore to be independent of the vascular lesions and not analogous to the myocarditis associated with arterial diseases in man.

Other possible explanations are based on a direct toxic action of adrenalin on the heart muscle, or an indirect action through the mechanical disturbances of the circulation which it causes. Although the physiological action of adrenalin on smooth muscle is well known we have no knowledge of its toxic action on the various types of muscle cells. Degenerative lesions in the parenchymatous cells and in muscle cells other than those of the heart and aorta are rarely seen, and it seems improbable that the toxic principle of adrenalin, if it exists, should be so selective as to affect only the muscle cells of the heart and the smooth muscle of the aorta and its larger branches. Indeed the limitation of degenerative lesions to the heart and larger blood vessels, and their practical absence in all other tissues, speak against any toxic action and point to an influence of a mechanical nature affecting these structures alone.

The action of adrenalin in constricting the arterioles with consequent increase in blood pressure is well known. This increase of blood pressure is alone not sufficient to explain the lesions, for if it were, we would expect the vascular changes due to adrenalin to be more general. But it is admissible to conceive of a temporary

ischæmia of terminal vascular territories, due to the direct action of the drug upon the smooth muscle cells of the coronary arterioles, which lasts a sufficient length of time to cause some interference with the nutrition of the affected areas. From physiological experiments this period would appear to continue about ten to fifteen minutes. If to this we add the increased contractile effort of the heart necessary to overcome the greatly augmented intravascular tension, we have factors sufficient to explain the degenerative lesions of the early experiments. The heart muscle under conditions of poor nutrition is pushed to its extreme power and dilatation results with injury of single fibres or groups of fibres. This injury is due in part to nutritive and in part to mechanical disturbance. That the dilatation is extreme, that the muscle is anæmic, and that serious alterations of the fibres are associated with this condition after but a single injection of adrenalin has been repeatedly demonstrated during the course of this investigation.

Practically the same explanation was advanced by Dr. Stanton and myself to explain the necrosis in the lesions of the aorta due to adrenalin. K. Ziegler's explanation of the vascular lesions is also very similar. He points out that the inner and middle portions of the media of the aorta, the usual seat of necrosis, is the part of the vessel most scantily supplied by the vaso-vasorum and therefore most susceptible to necrosis at the time of vascular spasm and local anæmia.

Comparison with Other Experimental Lesions of the Myocardium.—The degenerative changes due to adrenalin do not conform in all respects to those caused by other agents used experimentally. The hyaline and granular changes in the muscle fibres, the alterations in striation, and the slight accumulations of lymphoid cells are similar to those found by various investigators (Comba,[4] Welch and Flexner,[5] and Mollard and Regaud[6]) who have studied the effect of diphtheria toxin on the heart muscle. On the other hand they differ in the absence of fatty changes, in the extreme œdema of the interstitial tissue, and in the disorganization of the fibres. In the same way also they differ from the lesions produced by Welch[7] in his experimental study of the effect of high

temperature and from those produced by Ribbert[8] with Staphylococcus pyogenes aureus, by Charrin[9] with Bacillus pyocyaneus, by Tallquist[10] with Streptococcus pyogenes, by Björksten[11] with various bacteria or their toxins, and by Flexner[12] with ricin and abrin.

With the exception of the slight focal increase of connective tissue described by Charrin, Comba, and Mollard and Regaud, as a rare result of repeated injections of bacterial toxins, the only form of fibrous myocarditis mentioned in the literature of experimental lesions of the heart is that following ligation of the coronary arteries. The histological changes following such ligation have been described by Kolster, Porter, and Baumgarten[13]. The last, in his very detailed description, states that by the fourteenth day the new connective tissue had largely replaced the widespread areas of anæmic necrosis. This is of interest in connection with the lesions above described, in that the most marked connective tissue proliferation occurred in a rabbit killed sixteen days after the first injection.

Relation to Lesions in Man.—No definite comparisons can be made. The animals with acute degeneration of the myocardium and extreme dilatation of the heart which die suddenly after the fourth or fifth injection represent a condition apparently similar to that occasionally seen in man, where unusual strain causes sudden death due to an acute dilatation of a heart previously injured by some toxic agent or disturbance of nutrition. The later fibrous lesions have no analogy to the commoner chronic forms of myocarditis in man associated with arteriosclerosis or following known toxic conditions. That some of the more unusual forms of fibrous myocarditis in man which are difficult of explanation may be due to circulatory disturbances of the same general nature as those caused by adrenalin injections in the rabbit cannot be denied. It is to these only that the results of this experimental investigation appear to have any relation.

EXPLANATION OF PLATE XX.

Fig. 1.—Fibrous myocarditis with more or less focal arrangement of the new tissue. Rabbit No. 22; see description in text.

Fig. 2.—Higher magnification of a different portion of the same section show-

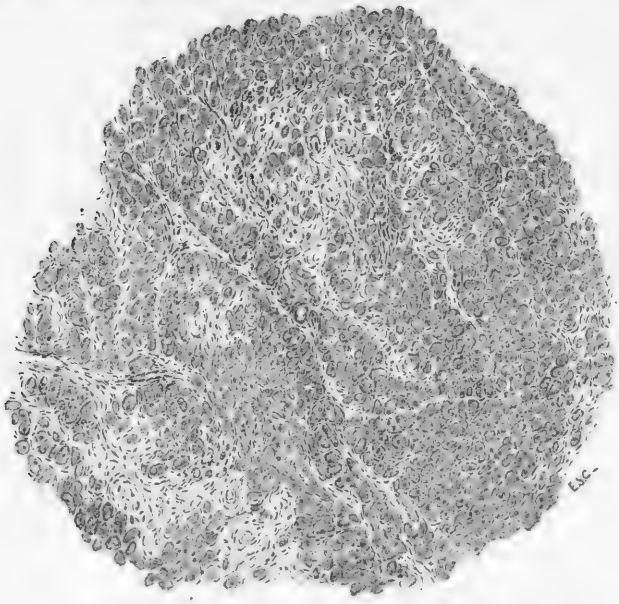

Fig. 1.

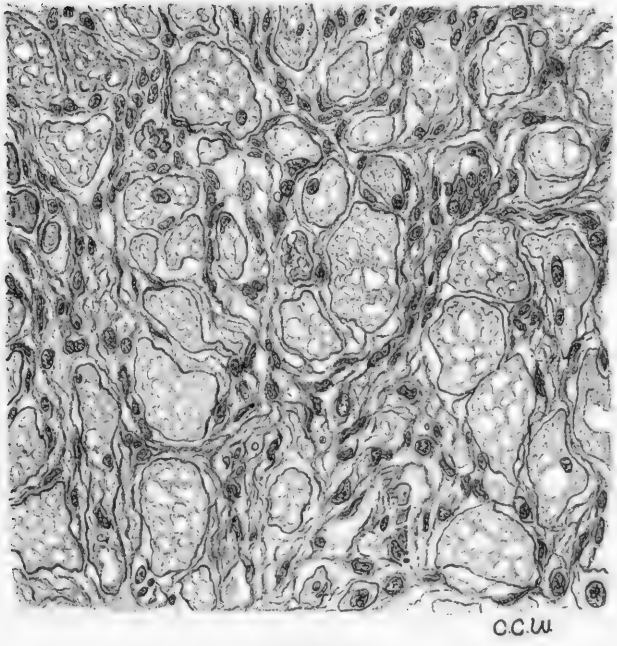

Fig. 2.

ing the character of the muscle degeneration and also a moderately diffuse increase of connective tissue.

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THE ENZYMES IN PHAGOCYTIC CELLS OF INFLAMMATORY EXUDATES.

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Those who have insisted upon the predominant importance of phagocytosis in the mechanism of natural and acquired immunity have not deserved Buchner's reproach that multiplied observation of this phenomenon can afford no explanation of its significance. Innumerable experiments have been divised to determine the conditions under which cells of various kinds ingest micro-örganisms and other substances and the fate of the ingested material. The conditions under which phagocytosis occurs are now fairly well understood, but less is known concerning intracellular digestion.

Metschnikoff[1] has shown that a close relation exists between phagocytosis in higher animals and the intracellular digestion of food by amœbæ, planarians, actinidæ, and other animals of relatively simple structure. Mouton[2] has studied the proteolytic enzyme which can be extracted with glycerine from the bodies of amœbæ and has found that it liquefies gelatin and digests feebly coagulated egg albumen; it acts both in a weakly acid and in a weakly alkaline medium. From the phagocytic cells which digest the food of actinidæ, or sea anemones, Krukenberg[3] and Mesnil[4] have obtained a similar enzyme which the latter has named actinodiastase. By analogy with the intracellular digestion of these invertebrates Metschnikoff has attempted to explain the process which occurs within phagocytic cells of vertebrates.

Common to all mammalian species are two types of cell capable of ingesting solid particles. One type is the polynuclear leucocyte

[1] L'immunité dans des maladies infectieuses, Paris, 1901.
[2] *Annal. de l'Inst. Pasteur*, 1902, xvi, 457.
[3] *Untersuchungen a. d. physiol. Inst. d. Univ. Heidelberg*, 1878, ii, 273.
[4] *Annal. de l'Inst. Pasteur*, 1901, xv, 352.

(polymorphonuclear) with fine granulation, the neutrophile leucocyte of human blood, which leaves the blood vessels and forms the chief cellular element of most inflammatory exudates. A second type of phagocytic cell is larger than the polynuclear leucocyte and has usually a single large nucleus which is round or somewhat irregular in outline; its protoplasm does not contain granules exhibiting a specific reaction to dyes. The polynuclear leucocyte with specific granulation is readily recognizable either within or without the blood vessels and histological and experimental studies have shown that it is derived from cells with similar specific granulation which divide by karyokinetic division in the bone marrow. The characters of the large mononuclear phagocyte are less distinctive and the identity of cells of this type occurring in different situations under different conditions has been much disputed. Such large mononuclear phagocytes, which Metschnikoff has called macrophages to distinguish them from the smaller polynuclear cells or microphages, are always found in inflammatory exudates which have accumulated in serous cavities; during the late stages of the inflammatory process they are actively engaged ingesting and destroying red blood corpuscles, polynuclear leucocytes, and other cellular elements. The large mononuclear phagocytes of the spleen, lymphatic glands, and other organs which during typhoid fever ingest red blood corpuscles and lymphocytes are doubtless similar in character.

The two types of phagocytic cells react differently to different substances. Polynuclear leucocytes it is well known are attracted by almost all kinds of bacteria and are especially concerned in the inflammatory reaction which follows the invasion of these micro-organisms. When for example Staphylococcus pyogenes aureus, Bacillus pyocyaneus, or Bacillus coli is injected into the peritoneal cavity of the guinea-pig in quantity insufficient to cause its death they are quickly ingested by polynuclear leucocytes; a few bacteria are taken up by mononuclear phagocytes. When, however, cellular elements such as red blood corpuscles or spermatozoa are injected into the peritoneal cavity of this animal they are as Metschnikoff [5] has shown attacked and

[5] *Annal. de l'Inst. Pasteur*, 1899, xiii, 737.

ingested by large mononuclear phagocytes or macrophages. Phagocytosis of certain parasites belonging to the animal kingdom, such as the malarial parasites and trypanosomes (Laveran and Mesnil [6]), is accomplished in great part at least by the same cells. The spirochæta of relapsing fever (Sawtschenko and Melkich[7]), and the similar parasite of geese (Metschnikoff [8]), organisms of which the zoölogical position is as yet undefined, suffer the same fate when injected into an insusceptible animal. It is noteworthy that certain bacteria, the bacillus of tuberculosis and the bacillus of leprosy (Iwanow [9]), are subject to phagocytosis by mononuclear cells, attracting in less degree polynuclear phagocytes.

It has long been known that intracellular digestion by amœbæ and other protozoa occurs in the presence of acid. Granules of litmus ingested by some of these organisms assume a red color and alizarin-sulphate and other indicators of reaction show that the contents of the digestive vacuoles is acid. Similar observations have shown that the phagocytic cells which form the digestive cavity of certain low animal species such as sponges, planaria, and actinians, and by intracellular digestion prepare food for digestion, secrete acid into digestive vacuoles surrounding particles of food. Various substances ingested by such phagocytic cells assume in the presence of neutral red a brownish red color which according to Metschnikoff indicates the presence of acid. When phagocytic cells of the higher animals are allowed to ingest granules of litmus or tournesol-blue no change occurs; alizarin-sulphate is unaffected, for the reason, as Metschnikoff suggests, that it has a toxic action upon the phagocytic cell. When, however, a drop of exudate from a guinea-pig which has received in the peritoneal cavity nucleated red blood corpuscles from a bird is treated with a weak solution of neutral red, the partially digested corpuscles, or nuclei of corpuscles, which have been engulfed by macrophages assume the brownish red color which

[6] *Annal. de l'Inst. Pasteur*, 1901, xv, 673.
[7] Ibid., 1901, xv, 497.
[8] L'immunité dans les maladies infectieuses, p. 169.
[9] *Annal. de l'Inst. Pasteur*, 1902, xvi, 705.

Metschnikoff attributes to a feebly acid reaction. J. Plato,[10] who first studied the effect of neutral red upon material ingested by phagocytes, found that bacteria are stained only after they are taken up by leucocytes and lose their color when the cell dies; he attributes the changes to oxidation and reduction of the dye, which with loss of oxygen becomes colorless. Himmel[11] has confirmed the observations of Plato; the reaction indicates, he believes, that intracellular digestion of bacteria occurs in the presence of a feebly acid reaction. Bacillus tuberculosis fails to exhibit this reaction even when ingested by the phagocytes of an insusceptible animal though other related bacteria which resist decolorization with acid assume a red color under the same conditions. Metschnikoff believes that intracellular digestion of bacteria usually occurs in the presence of a feebly acid reaction but may, as with the tubercle bacillus, occur in a weakly alkaline medium. The experiments which will be described do not support this opinion.

It is unnecessary to review the arguments with which Buchner, Metschnikoff, and others have maintained that the alexine or complement or cytase of the serum is contained in the leucocytes from which it is set free, according to Buchner by a process of secretion, or according to Metschnikoff by disintegration of cells (phagolysis), nor to consider the views of those who have opposed these theories. Buchner has suggested that the alexine is of the nature of an enzyme and not improbably is identical with the proteolytic ferment which is present within the leucocytes. Metschnikoff has claimed that the disintegration of red blood corpuscles which occurs within the macrophage is analogous to that which is produced by proteolytic enzymes within the digestive vacuoles of amœbæ and phagocytic cells of low invertebrate species. The enzyme or cytase which accomplishes intracellular digestion of bacteria is he believes identical with the body discovered in the serum by Nuttall and named by Buchner alexine but later more accurately defined by Ehrlich and designated complement. The observations which Metschnikoff has

[10] *Arch. für mikros. Anat.*, 1900, lvi, 868.
[11] *Annal. de l'Inst. Pasteur*, 1902, xvi, 663.

brought forward in support of his belief that the macrophages contain macrocytase or hæmolytic complement while the polynuclear leucocytes or microphages contain a second enzyme-like body, microcytase or bactericidal complement, will not be cited. Analogy with intracellular digestion among lower forms of animal life make it not improbable according to Metschnikoff that these bodies have the nature of proteolytic enzymes.

The occurrence of proteolytic enzymes in the cells of purulent exudates has been demonstrated by Fr. Müller, whose observations are recorded by Kossel.[12] A glycerine extract of purulent phthisical sputum was found to digest fibrin and coagulated albumen in a weakly alkaline medium. Other purulent sputa have the same power but purely mucous sputa do not exhibit it. Leber[13] has demonstrated the presence of proteolytic enzymes in purulent exudates and Achalme[14] has shown that they may liquefy gelatin and dissolve fibrin, coagulated egg albumen, and casein. By means of the Kjeldahl method, determining before and after digestion the amount of nitrogen contained in substances precipitable by tannic acid, Ascoli and Mareschi[15] have shown that the sediment from a sterile exudate obtained by the injection of aleuronat undergoes autolysis under toluol. Autolysis does not occur when the sediment has been subjected to a temperature of 60° C. during three hours.

Umber,[16] employing the method of Kjeldahl, found that the peritoneal fluid from two patients suffering with abdominal tumor underwent autolysis, but Schütz[17] found by the same method no measurable destruction of proteids though the exudates which he used contained in two instances a considerable number of leucocytes. Zak[18] studied pleural and peritoneal exudates from twelve patients. He found no autolysis in half of the cases and

[12] *Zeit. für klin. Med.*, 1888, xiii, 149.

[13] Ueber die Enstehung der Entzündung, Leipzig, 1891.

[14] *Compt. rend. de la Soc. de Biol.*, 1899, li, 568.

[15] Eleventh Italian Congress for Internal Medicine, Pisa, October 27–31, 1901. Ref., *Maly's Jahresber.*, 1902, xxxii, 291.

[16] *Münchener med. Woch.*, 1902, xlix, 1169.

[17] *Zent. für inner. Med.*, 1902, xxiii, 1161.

[18] *Wiener klin. Woch.*, 1905, xviii, 376.

could demonstrate no relation between the abundance of cells and the degree of autolysis. A somewhat similar observation has been made by Hahn,[19] who has found that an artificially produced pleural exudate does not cause liquefaction of gelatin unless several days have elapsed since the injection of the inflammatory irritant.

Experiments which I have described in a previous publication[20] have explained the discrepant observations just cited. The serum of the inflammatory exudate and of the blood inhibits the activity of the proteolytic enzymes contained in the leucocytes. When the leucocytes of the exudate are separated from the serum they undergo autolysis and are capable of digesting actively foreign proteid. In the late stages of inflammation produced by the injection of aleuronat into the pleural cavity of dogs there is some diminution of the anti-enzymotic action of the serum of the exudate.

Methods.—The methods employed have been described in the article to which reference has just been made. Exudates rich in leucocytes have been obtained by injecting a suspension of aleuronat in water containing starch (aleuronat, 5 grm.; starch, 1.5 grm., water, 100 c.c.) into the pleural cavities of dogs. Since the serum of the exudate inhibits the activity of the proteolytic enzymes contained in the leucocytes, it has been necessary to separate carefully cells from serum by repeated centrifugalization with normal salt solution (0.85 per cent.). In order to compare the activity of enzymes contained in leucocytes of different exudates the washed cells were measured in a graduated centrifugal tube after centrifugalization and suspended in a definite quantity of salt solution—equal to nine (1 : 10), nineteen (1 : 20), or twenty-nine (1 : 30) times the volume of the cells which were obtained. A measured quantity of the suspension of leucocytes was allowed to act upon heated blood serum, which being readily obtained formed a convenient means for testing proteolytic enzymes. Blood serum of the dog or of the ox mixed with an equal volume of salt solution does not coagulate, though it becomes

[19] Handbuch der pathogenen Mikroorganismen, herausgeg. von Kolle u. Wassermann, Jena, 1904, iv, 281.

[20] *Jour. of Exper. Med.*, 1905, vii, 316.

opaque, when heated to 75° C. for one half hour, and may be accurately measured in a pipette. At this temperature the proteolytic enzymes of the serum described by Delezenne and Pozerski [21] and by Hedin,[22] as well as its anti-enzymotic action, are wholly destroyed.

In all of the experiments to five cubic centimeters of heated serum (ten cubic centimeters of the diluted serum) were added in a small sterilized flask five cubic centimeters of the suspension of cells to be tested. Normal salt solution in sufficient quantity to bring the entire volume to twenty-five cubic centimeters was added, acid or alkali being substituted for salt solution in sufficient quantity to make any desired strength. After adding one cubic centimeter of toluol the flask was securely closed with a rubber stopper and kept at 37° C. in most instances during five days.

The same quantities of cells and of serum used for digestion were coagulated by heating to boiling and the nitrogen in uncoagulable form determined by the Kjeldahl method. The difference between the nitrogen of uncoagulable substances present in a flask kept at 37° C. and that of the control coagulated immediately represents the coagulable proteid converted by digestion into uncoagulable form. For the sake of convenience nitrogen determined by the Kjeldahl method will be represented by cubic centimeters of $\frac{1}{10}$ N sulphuric acid, since for comparison of results it is unnecessary to convert these figures into the corresponding quantities of nitrogen.

In the earlier experiments much difficulty was experienced in accurately coagulating the undigested proteid, complete coagulation being dependent upon a reaction which varies with the quantity of proteid. The method finally adopted was as follows: To each flask containing twenty-five cubic centimeters of fluid was added an equal volume of a sixteen per cent. solution of magnesium sulphate (20 grm. to 100 c.c. of water). After heating to boiling, acetic acid in sufficient quantity to react with litmus was added. After boiling, a weak solution of sodium hydroxide

[21] *Compt. rend. de la Soc. de Biol.*, 1903, lv, 327, 690, 693.
[22] *Jour. of Physiol.*, 1904, xxx, 195.

was added in such excess that on boiling again the coagulated proteids formed a flocculent coagulum which after cooling subsided and left the fluid perfectly clear. Since an excess of sodium hydroxide combines with magnesium sulphate to form insoluble magnesium hydroxide, the reaction of the solution is thus made approximately neutral.

In a publication previously cited I have shown that the enzymes contained in the leucocytes of an inflammatory exudate are capable of digesting proteid both in an alkaline and in an acid medium, though their activity is greater in the former. Hedin and Rowland [23] had shown that autolysis of parenchymatous organs, such as the spleen, kidneys, liver, heart, and lymphatic glands, occurs more actively in an acid than in an alkaline medium. From the spleen Hedin[24] has succeeded in isolating two enzymes, one of which acts with the aid of acid, the other in the presence of alkali. I have shown [25] that the bone marrow resembles the cells of an inflammatory exudate in its action on proteids, digesting far more actively in an alkaline than in an acid medium, and differs from other organs such as the liver, spleen, kidney, and lymphatic glands of which the proteolytic action is stronger in an acid medium. Since histologists with few exceptions are agreed that the polynuclear leucocytes with fine granulation are derived from the bone marrow, proteolysis produced in an alkaline medium by these cells may be referred to an enzyme formed in the bone marrow. The purpose of the following experiments has been to determine if leucocytes of the inflammatory exudate contain one enzyme which acts both in an acid and in an alkaline medium, or two enzymes one of which, like pepsin, acts in the presence of acid while the other, resembling trypsin, acts in the presence of alkali.

Preliminary Experiments.—Since the exudates obtained by the injection of aleuronat into the pleural cavities of the dog frequently, contain red blood corpuscles which are present in considerable number especially when the injection has been

[23] *Zeit. für physiol. Chem.*, 1901, xxxii, 341, 531.
[24] *Jour. of Physiol.*, 1904, xxx, 155.
[25] *Jour. of Exper. Med.*, 1905, vii, 759.

accompanied by injury to the lung, it was considered necessary to test the action of red blood corpuscles on heated serum and to exclude the possibility that they contain enzymes capable of causing proteolysis either in an acid or in an alkaline medium. The following experiment shows that the proteolytic action of these cells is insignificant.

EXPERIMENT I.—Mixtures containing 5 c.c. heated serum and 5 c.c. of a suspension of red blood corpuscles washed three times by centrifugalization and suspended in salt solution (in dilution of 1:20) were kept at 37° C. for five days.

With 0.2% acetic acid	3.05 c.c.
Without addition	2.3 "
With 0.2% sodium carbonate	2.45 "
Control	2.15 "

In the experiments which I have recorded in previous articles the ability of the enzymes of the exuded leucocytes to digest proteids in a medium made acid by acetic acid was tested. In order to exclude the possibility that proteolysis occurs only with acetic acid, other acids, namely hydrochloric and sulphuric, were used. By adding two and a half, five, or ten cubic centimeters of the $\frac{1}{10}$ N acids to each of a number of flasks containing five cubic centimeters of a suspension of leucocytes together with five cubic centimeters of heated serum and bringing the total volume of each flask to twenty-five cubic centimeters with normal salt solution, the strength of the acid present was made respectively $\frac{1}{100}$, $\frac{1}{60}$, and $\frac{1}{5}$ N. For the sake of comparison flasks containing sodium carbonate in the same strength were prepared.

EXPERIMENT II.—Leucocytes from an exudate removed from the pleural cavities of a dog five days after the injection of aleronat were suspended in nineteen times their volume (1:20) of salt solution. The following figures represent in cubic centimeters of $\frac{1}{10}$ N sulphuric acid the amounts of nitrogen in substances uncoagulable by heat present in 5 c.c. suspension of cells and 5 c.c. heated serum after digestion five days at 37° C. in the media which have been mentioned:

	Acetic Acid	Sulphuric Acid	Hydrochloric Acid	Sodium Carbonate
$\frac{1}{25}$ N.	21.15 c.c.	5.25 c.c.	4.4 c.c.	20 35 c.c.
$\frac{1}{10}$ N.	18.95 "	18.2 "	13.95 "	28.05 "
$\frac{1}{100}$ N.	—	20.7 "	17.9 "	29.05 "

Digestion without addition of acid or alkali	24.55 c.c.
Control	4.0 "

It is noteworthy that the maximum digestion occurs in a slightly alkaline medium. When no addition has been made the normal alkalinity of the heated serum though diluted with four times its volume of fluid gives slight alkalinity to the medium. The efficiency of the three acids in favoring proteolysis varies considerably; sulphuric and hydrochloric acids in the same strength ($\frac{1}{25}$)N in which acetic acid favors digestion almost wholly prevent it, while in much weaker solution they cause active digestion. If, as it is not improbable, the maximum efficiency of hydrochloric acid is represented by a strength less than $\frac{1}{100}$ N the relative activity of the three acids follows the same order as their dissociation.

Preservation of Proteolytic Enzymes of Leucocytes by Glycerine.—In the experiments just described as well as in those recorded in previous publications the enzymotic action of cells has been tested immediately after removal from the animal. For certain experiments preservation of the enzymes was desirable and several methods of preserving them were attempted. Suspensions of cells in fifty per cent. glycerine preserved their ability to digest proteid in an acid as well as in an alkaline medium; proteolysis produced by the suspension in glycerine was only slightly less than that caused by the same cells diluted to the same extent with salt solution and used in the fresh state.

Experiment III.—Leucocytes from a dog killed five days after the injection of aleuronat into the pleural cavity were suspended in nine times their volume of salt solution (1:10). To a part of this suspension was added an equal volume of salt solution and the ability of the diluted suspension (1:20) to digest heated serum tested in the presence of 0.2% acetic acid, in 0.2% sodium carbonate, and with an unchanged reaction. To the remainder of the original suspension was added an equal volume of glycerine, making the dilution of the suspension 1:20. At the end of a week and again at the end of a month the action of 5 c.c. of the suspension was tested under conditions similar to those previously employed. In all instances digestion occurred during five days at 37° C.

	Suspension of fresh cells	Suspension in glycerine after 1 wk.	Suspension in glycerine after 1 mo.
With 0.2% acetic acid	20.9 c.c.	17.5 c.c.	18.8 c.c.
Neutral	26.0 "	—	26.55 "
With 0.2% sodium carbonate	28.65 "	26.95 "	29.8 "
Control	2.9 "	4.0 "	6.8 "

At first sight the figures suggest that the activity of the enzyme has at the end of a month increased rather than diminished. It may be noted, however, that there is a corresponding increase in the figure representing the control, doubtless as the result of slow autolysis occurring in the glycerine extract kept at room temperature. When the figure representing the control is subtracted from that obtained at the end of digestion it is found that in an acid medium digestion is less active when the glycerine extract is used (13.5 c.c.) than with the fresh suspension (18 c.c.); activity of the enzyme has diminished slightly at the end of a month (12 c.c.). In an alkaline medium the glycerine extract is somewhat less active (22.95 c.c.) than the fresh suspension (25.75 c.c.) and has remained unchanged during a month (23 c.c.).

Preservation of Proteolytic Enzyme of Leucocytes by Drying after Treatment with Alcohol and Ether.—The attempt was made to determine if leucocytes dried and reduced to a powder retain their enzymotic action. The cells, obtained from an exudate by centrifugalization, after repeated washing with many times their volume of salt solution were treated with about fifteen times their volume of absolute alcohol; part of the alcohol was poured off after the cells had settled and replaced with fresh absolute alcohol. After standing about an hour the alcohol was removed by filtration and replaced with ether. After a short time the ether was removed by filtration and compression between pads of filter paper. The cells, allowed to dry in the air, were reduced in a mortar to a fine powder, which on account of the presence of a small amount of red blood corpuscles had a dull red color.

EXPERIMENT IV.—Leucocytes were obtained from a dog eight days after the injection of aleuronat into the left pleural cavity and dried by the method described. The dried powder (20 mgr.) was allowed to act upon heated serum (5 c.c.) in acid, neutral, and alkaline media for five days at 37° C.

With 0.2% acetic acid	7.55 c.c.
Without addition	22.8 "
With 0.2% sodium carbonate	18.8 "
Control	5.8 "

EXPERIMENT V.—Powdered leucocytes (20 mgr.) obtained from a dog two days after the injection of aleuronat acted on heated serum eight days at 37° C.

With 0.2% acetic acid	5.5 c.c.
Without addition	19.05 "
With 0.2% sodium carbonate	12.55 "
Control	2.45 "

EXPERIMENT VI.—The proteolytic activity of the dried enzyme from an exudate obtained twenty-four hours after the injection of aleuronat was tested four and a half months later with the following result:

With 0.2% acetic acid	6.3 c.c.
Without addition	22.85 "
With 0.2% sodium carbonate	21.1 "
Control	5.35 "

The powder prepared from leucocytes of a sterile exudate digests proteid actively in an almost neutral and in an alkaline medium but has almost completely lost the power of the fresh cells to digest in an acid medium. The most probable explanation of this fact is that the leucocytes of the exudate contain two ferments, one of which digests in a neutral or slightly alkaline medium, while the other acts in an acid medium; the former is uninjured during the process of preparing the dry powder, while the latter is destroyed. The enzyme which acts in an alkaline medium has preserved its activity unchanged more than four months in the dry condition.

For comparison with results to be described later an experiment showing the effect of various quantities of the enzyme upon a measured quantity of proteid is recorded.

EXPERIMENT VII.—Various quantities of the powder used in Experiment VI were allowed to act during five days at 37° C. upon 5 c.c. heated serum without addition of acid or alkali.

With 10 mgr. powder	19.05 c.c.
" 20 " "	22.8 "
" 50 " "	26.05 "
Control	5.8 "

The figures only very roughly indicate the velocity of the reaction produced by various quantities of ferment but show that a considerable increase in the amount of the latter causes a moderate increase in the amount of proteolysis at the end of a given time. E. Schütz,[26] studying by polarimetric estimation

[26] *Zeit. für physiol. Chem.*, 1885, ix, 577.

the amount of pepton formed from egg-white by the action of pepsin, reached the conclusion that the rapidity of digestion is proportional to the square root of the quantity of enzyme. Using Mett's tubes, in which a measured column of coagulated albumen is subjected to digestion, Borissow[27] and Samojloff[28] have obtained similar results. J. Schütz[29] has found that digestion may be shown to follow this rule by estimating by the Kjeldahl method the nitrogen of coagulable proteid converted into uncoagulable form. That the same rule is applicable to tryptic digestion has been maintained by Pawlow[30] and by Vernon.[31] For the present purpose the mathematical accuracy of these statements is not significant ; it is sufficient to point out that only a slight increase in the degree of proteolysis may be caused by large increase in the amount of enzyme.

Effect of Temperature on the Proteolytic Enzymes of Leucocytes.—Further evidence that the leucocytes of the inflammatory exudate contain two enzymes is furnished by the different effect of various temperatures upon proteolysis when tested in an acid and when tested in an alkaline medium. For this purpose suspensions of fresh cells were used; after five cubic centimeters of the suspension of cells in salt solution had been subjected to various temperatures for one half hour, five cubic centimeters of heated serum were added together with acid or alkali in quantity to bring the entire amount of fluid (25 c.c.) to a strength of 0.2 per cent.

EXPERIMENT VIII.—Leucocytes from an exudate removed from the pleural cavity of a dog twenty-three hours after the injection of aleuronat were suspended in nineteen times their volume of salt solution (1:20). The result of digestion caused by this suspension of cells heated to various temperatures was as follows:

	In 0.2% acetic acid	In 0.2% sodium carbonate
Leucocytes unheated	13.65 c.c.	24.05 c..c
" heated at 50° C.	13.5 "	26.0 "
" " " 55° C.	11.2 "	27.4 "
" " " 60° C.	8.7 "	27.45 "
" " " 65° C.	9.95 "	25.65 "
" " " 100° C.	4.0 "	4.75 "

[27] Inaug. Diss., St. Petersburg, quoted by Pawlow.

[28] *Pflüger's Archiv*, 1901, lxxv, 86.

[29] *Zeit. für physiol. Chem.*, 1900, xxx, 1.

[30] The Work of the Digestive Glands (Trans.), London, 1902.

[31] *Jour. of Physiol.*, 1901, xxvi, 405.

EXPERIMENT IX.—Leucocytes obtained twenty-four hours after the injection of aleuronat were subjected to various temperatures as before.

	In 0.2% acetic acid	In 0.2% sodium carbonate
Leucocytes unheated	16.6 c.c.	30.2 c.c.
" heated at 55° C.	14. "	32.85 "
" " " 60° C.	12.45 "	— "
" " " 65° C.	12.1 "	— "
" " " 70° C.	8.65 "	29.65 "
" " " 100° C.	4.55 "	5.2 "

Temperatures between 55° and 70° C. diminish the enzymotic activity of the fresh leucocytes exhibited in an acid medium but do not materially diminish proteolysis in an alkaline medium. In an alkaline medium, indeed, the activity of proteolysis is slightly increased when the cells have been subjected to low temperatures. A somewhat similar observation has been made by Biernacki,[32] who found that heating trypsin in an alkaline solution in the presence of certain salts or of albumoses at 45° C. during from five to ten minutes increased its proteolytic activity so that digestion proceeded more rapidly than was usual.

Though the power to digest in an acid and in an alkaline medium is differently affected by temperatures below 70° C., proteolysis in both media is prevented by higher temperatures.

EXPERIMENT X.—Leucocytes removed from the pleural cavity of a dog five days after injection of aleuronat were suspended in nineteen times their volume of salt solution (1:20). Power to digest heated serum was tested in acid and in alkaline media after heating to temperatures between 70° and 100° C.

	In 0.2% acetic acid	In 0.2% sodium carbonate
Leucocytes unheated	16.35 c.c.	25.0 c.c.
" heated at 70° C.	4.75 "	4.25 "
" " " 75° C.	3.05 "	3.6 "
" " 80° C.	3.65 "	3.75 "
" " " 100° C.	2.65 "	3.65 "

Although in this experiment the effect of heating at 70° C. is greater than in the two preceding experiments, it is evident that enzymotic activity of fresh cells is wholly destroyed at a temperature between 70° and 75° C. This statement applies only to the conditions of the experiment, since Biernacki has shown that reaction, and the presence of certain salts or of albumoses affect the

[32] *Zeit. f. Biol.*, 1891, xxviii, 49.

ability of trypsin and pepsin to withstand various temperatures.

Changes in the Proteolytic Enzymes of Cells of Inflammatory Exudates Occurring during the Progress of Inflammation.—When aleuronat is injected into the pleural cavity of the dog, as Buchner first showed, an active inflammatory reaction ensues and leucocytes together with a large quantity of serum accumulate in the cavity. At the end of from fifteen to twenty hours after injection of from five to ten cubic centimeters of a suspension of aleuronat there is a considerable quantity, in some cases more than one hundred cubic centimeters, of thin grayish-pink or red fluid containing usually not more than five per cent. of cells (measured after centrifugalization) among which red blood corpuscles are often numerous. Aleuronat is no longer free in the fluid but is firmly adherent to the pleural surfaces, accumulating along the anterior mediastinum just above the diaphragm. The lymphatic glands in the mediastinum, particularly those behind the upper part of the sternum, enlarge rapidly. After the injection of ten cubic centimeters of the suspension of aleuronat into each pleural cavity the latter usually contain at the end of from three to five days or even longer a considerable amount of fluid which is now much more turbid and richer in cells. Resolution of the exudate occurs, the aleuronat is absorbed, and except for the presence of fibrous adhesions the cavities return to their normal condition.

At the end of from sixteen to twenty-four hours after the injection polynuclear leucocytes with fine granulation form the greater number of the cells which are present. Differential counts show that from eighty to ninety per cent. of the leucocytes are of this type. The remainder of the cells are of the mononuclear type but few lymphocytes are present. The greater number of these mononuclear cells are somewhat larger than polynuclear leucocytes and have a large vesicular nucleus, which is usually somewhat irregular in outline, and fairly abundant protoplasm. They have the characters of the large mononuclear leucocytes of the blood. Since they are capable of active phagocytosis Metschnikoff has designated them macrophages; Kanthack and Hardy [33]

[33] *Jour. of Physiol.*, 1894, xvii, 81.

and other English writers have called them hyaline cells; Marchand [34] describes them as leucocytoid cells.

During the later stages of the inflammatory process polynuclear leucocytes with fine granulation are very numerous but their percentage is somewhat diminished since the large mononuclear cells have increased in number. Leucocytes with eosinophile or basophile granulation are present in insignificant number. At the end of three or five days large mononuclear cells form from twenty-five to thirty-five per cent. of the cells; the large proportion noted by Tarassévitch,[35] namely fifty to sixty per cent. two or three days after the injection of aleuronat into the pleural cavities of rabbits, was not found. The percentage counts of large mononuclear cells give little indication of the changes which they undergo, since as the exudate increases in age they increase in volume so that at the end of three or more days there are many cells with a diameter four or five times that of a polynuclear leucocyte. At this time very active phagocytosis is in progress and mononuclear cells with vacuolated protoplasm may contain red blood corpuscles or polynuclear leucocytes in various stages of disintegration; one large phagocyte may contain six or more of such cells. Mononuclear cells at this time are often clumped together to form masses which may contain from ten to fifty cells. The inflammatory reaction which follows the injection of aleuronat does not differ materially from that produced by the injection of various bacteria (described by Durham [36]) or by the injection of carmine, starch, or lycopodium powder (described by Marchand [37]) into guinea-pigs and other animals.

Since the cellular character of the exudate changes during the course of inflammation opportunity is afforded to study the relation between the proteolytic enzymes which are present and the types of phagocytic cells which have been described. For this purpose the power of a measured quantity of cells to digest a measured quantity of proteid has been tested in acid, in neutral,

[34] *Verhandl. der deutsch. path. Gesellsch.*, 1898, i, 63.
[35] *Annal. de l'Inst. Pasteur*, 1902, xvi, 127.
[36] Loc. cit.
[37] Loc. cit.

or in alkaline media. In the records which follow the amounts of digestion represented in cubic centimeters of $\frac{1}{10}$ N sulphuric acid have been obtained by subtracting the figure representing the control, coagulated immediately, from that representing the amount of nitrogen in substances uncoagulable by heat present after digestion for five days at 37° C. Differential counts of the leucocytes contained in the exudate have been made. Since cells with eosinophile or basophile granules have been rarely encountered, by polynuclear leucocytes are designated cells with fine granulation, corresponding to the neutrophile leucocytes of human blood. Mononuclear cells include not only the large mononuclear phagocytes but smaller cells resembling the lymphocytes of the blood; the latter form only a very small proportion, rarely so much as two or three per cent. of the number of cells.

It has been desirable to separate the experiments into several groups since in all instances the conditions of the experiment have not been the same.

SERIES A.—In the following experiments inflammatory exudates were obtained by injecting 10 c.c. of suspension of aleuronat into each pleural cavity of dogs which were killed at the end of one, two, four, and five days respectively. The washed leucocytes were suspended in nineteen times (1:20) their volume of salt solution. At the time these experiments were performed the method of comparison finally adopted was not contemplated so that the record is in some instances incomplete. The following figures represent the digestion of 5 c.c. heated serum produced by 5 c.c. of cell suspension kept at 37° C. during five days (save in Experiment XII in which digestion was stopped at the end of four days) in 0.2% sodium carbonate, in an approximately neutral medium, and in 0.2% acetic acid.

Experiment	Age of exudate	Reaction of medium			Cells of exudate	
		Alkaline	Neutral	Acid	Polynuclear	Mononuclear
XI	1 day	19.7 c.c.		9.3 c.c.	84.8 %	15.2 %
XII	2 days	0.3 "		7.3 "	81.2 %	18.8 %
XIII	4 "	0.6 "	15.8 c.c.	10.15 "	73.8 %	26.2 %
XIV	5 "	21.85 "		13.2 "	78.2 %	21.8 %
XV	5 "	24.05 "	20.55 "	17.15 "	68.8 %	31.2 %

In Experiment XV (See Experiment II) digestion occurred in $\frac{1}{50}$ N sodium carbonate (approximately 0.2%) and in $\frac{1}{25}$ N acetic acid (approximately 0.24 %).

The ability of the fresh cells to digest in an alkaline medium shows considerable irregularity which has not been repeated in

subsequent experiments; the almost total absence of digestion in Experiments XII and XIII has not been explained. In only two experiments was the power to digest in a neutral medium tested. With proteolysis in an acid medium there was an almost uniform increase as the time between the injection of the inflammatory irritant and the removal of the exudate increased.

A second series of two experiments is not comparable with those just described because the cell suspensions used were prepared by suspending cells in nine times their volume of salt solution (1:10).

SERIES B.—In the two experiments which follow the conditions are the same as those in Series A, save that washed leucocytes were suspended in nine times their volume of salt solution.

Experiment	Age of exudate	Reaction of medium			Cells of exudate	
		Alkaline	Neutral	Acid	Polynuclear	Mononuclear
XVI	1 day	28.0 c.c.	16.95 c.c.	12.5 c.c.	84.0%	16.0%
XVII	5 days	26.7 "	18.55 "	17.85 "	73.6%	26.4%

Although the ability of the cells to digest in an alkaline or neutral medium has undergone little change, their efficiency in an acid medium has materially increased. At the same time the proportion of mononuclear cells has increased. It may be recalled on the one hand that the proportion of mononuclear cells does not accurately represent their relation to those with polymorphous nucleus since along with an increase in number the mononuclear phagocytes have undergone a great increase in volume, often having a diameter from three to five times as great as that of the polynuclear cells. On the other hand the amount of digestion during a given time does not increase in direct proportion to the amount of ferment contained in the cells but is more closely related to the square root of its quantity.

The exudates used in the preceding experiments were obtained by injecting a large amount of aleuronat, ten cubic centimeters of the suspension, into each pleural cavity. At the end of five days aleuronat is still present in great quantity adherent to the parietal pleura and embedded within the tissue of the anterior mediastinum. Doubtless as the result of the presence of the

foreign body in large amount irritation is continued and the exudate fails to undergo resolution; at the end of five days there may be present in each pleural cavity 150 or 200 cubic centimeters of fluid. In the following experiments a smaller quantity of aleuronat, five cubic centimeters of the suspension, was injected into the right pleural cavity alone in the hope that long continued exudation of polynuclear leucocytes might be avoided. In an animal killed at the end of four days the right pleural cavity contained 75 cubic centimeters of fluid in which however the proportion of polynuclear leucocytes was still large.

SERIES C.—Cells obtained one and five days after the injection of 5 c.c. of aleuronat suspension into the right pleural cavity of dogs were suspended in nineteen times their volume of salt solution (1:20).

Experiment	Age of exudate	Reaction of medium			Cells of exudate	
		Alkaline	Neutral	Acid	Polynuclear	Mononuclear
XVIII	1 day	26.35 c.c.	22.0 c.c.	10.05 c.c.	85.8%	14.2%
XIX	5 days	25.75 "	23.1 "	18.0 "	71.6%	28.4%

SERIES D.—In two experiments the conditions were identical with those just described save that washed leucocytes were suspended in twenty-nine times their volume of salt solution (1:30).

Experiment	Age of exudate	Reaction of medium			Cells of exudate	
		Alkaline	Neutral	Acid	Polynuclear	Mononuclear
XX	1 day	19.4 c.c.	16.95 c.c.	6.5 c.c.	87.6%	12.4%
XXI	4 days	17.2 "	20.6 "	12.9 "	75.8%	24.2%

The results of the experiments of Series B, C, and D are uniform; with the progress of the inflammatory reaction there is an increase in the proportion of mononuclear cells and diminution in the percentage of polynuclear leucocytes, while corresponding with these changes there is a slight diminution of proteolysis in an alkaline medium and a well marked increase in an acid medium. On the one hand the polynuclear leucocytes have decreased from approximately eighty-five to seventy-five per cent. and on the other hand the mononuclear cells have almost doubled in number and at the same time have increased much in bulk. Evidence has already been produced to show that the cells of inflammatory exudates contain two enzymes characterized like pepsin and tryp-

sin by their relation to acids and alkalis. The preceding experiments afford evidence that the polynuclear leucocytes contain that enzyme which is capable of active proteolysis both in an alkaline and in an almost neutral medium while the large mononuclear phagocytes produce that enzyme which digests proteid in an acid medium. It is not improbable that the latter enzyme produces slight proteolysis in a neutral medium since in Series B, C, and D there is with a marked increase of digestion in an acid medium a slight increase of digestion in a neutral medium.

Further evidence that the power to digest in an acid medium is dependent upon the mononuclear phagocytes is afforded by the following experiments in which were employed exudates produced by the injection of red blood corpuscles of the rabbit into the pleural cavity of the dog. Metschnikoff has shown that an exudate rich in mononuclear cells is obtained within twenty-four hours after the injection of red blood corpuscles of one species into an animal of another species. The large proportion of mononuclear cells noted by Gengou,[38] who found the exuded cells almost wholly of this type, was not obtained.

SERIES E.—Into each pleural cavity of two dogs were injected 5 c.c. of washed red blood corpuscles of the rabbit. In one animal, killed at the end of nineteen hours, the two cavities contained about 50 c.c. of deep red fluid from which 2.9 c.c. of cells were obtained by centrifugalization. In the two pleural cavities of the second animal, killed after three days, were 10 c.c. of thick pinkish-gray fluid from which were obtained 5.9 c.c. of cells. Suspensions of the leucocytes in both instances diluted in the proportion of 1:20 were allowed to act upon heated serum under the usual conditions.

Experiment	Age of exudate	Reaction of medium			Cells of exudate	
		Alkaline	Neutral	Acid	Polynuclear	Mononuclear
XXII	19 hours	23.9 c.c.	20.65 c.c.	14.3 c.c.	63.6%	36.4%
XXIII	3 days	21.65 " *	19.5 "	13.75 "	63.2%	36.8%

*Digestion occurred in presence of 0.1% sodium carbonate.

The previously observed increase of proteolysis in an acid medium associated with an increase in the age of the exudate has not occurred when the proportion of mononuclear phagocytes has not undergone noteworthy change.

Changes in the Proteolytic Activity of Cells of Lymphatic Glands

[38] *Annal. de l'Inst. Pasteur*, 1901, xv, 68.

near the Site of Inflammation.—The enlarged lymphatic glands which are situated in the neighborhood of the focus of inflammation afford further opportunity to study the enzymotic action of the large mononuclear phagocytes. Aleuronat injected into the pleural cavity of the dog rapidly accumulates, in part perhaps as the result of gravity, upon and within the anterior mediastinum, which at the end of from three to five days is converted into a thick firm mass of tissue within which aleuronat is still recognizable. Microscopical examination shows that masses of aleuronat are surrounded by polynuclear leucocytes which in the immediate neighborhood of the substance have not infrequently undergone necrosis. Mononuclear cells of the type previously described are numerous. In the anterior mediastinum above the level of the heart aleuronat is much less abundant, the tissue is moderately œdematous, and there are present at least a half dozen enlarged succulent lymphatic glands. Two larger than the others are situated immediately behind the upper part of the sternum and at the end of about three days after injection of the irritant measure one and a half centimeters in length.

Microscopical examination of these mediastinal lymphatic glands show that their sinuses are distended with fluid and contain even within twenty-four hours after the injection of aleuronat large mononuclear cells which do not differ from those found in the pleural exudate and are capable of ingesting red blood corpuscles and polynuclear leucocytes. These cells increase progressively in size and number and in some instances are so closely packed within the sinuses that the organ has the microscopic appearance of a mesentric lymphatic gland during typhoid fever. Not only within the sinuses but in the medullary cords as well are found such cells containing numerous partially disintegrated polynuclear leucocytes. In the sinuses and in the medullary cords free polynuclear leucocytes are numerous; the germinating centers of the follicles are enlarged and do not contain polynuclear leucocytes.

Since these lymphatic glands contain large mononuclear phagocytes massed together in large number it was thought not improbable that they would exhibit some change in their ability

to produce proteolysis. Jacoby [39] has shown that the thymus gland undergoes autolysis' and Hedin and Rowland [40] have found that autolysis of lymphatic glands proceeds more rapidly in an acid than in an alkaline medium. In a previous publication I have shown that an emulsion of cells prepared from the mesenteric lymphatic glands of the dog is capable of digesting heated blood serum.

By scraping the gland with a knife and forcing the tissue through a small seize made with fine wire gauze, cells were completely separated from stroma. After washing the cells with salt solution they were measured in a graduated centrifugal tube and suspended in a definite quantity of salt solution. Since the normal mediastinal glands of the dog are too small to furnish cells in sufficient quantity for use it was necessary to compare the proteolytic activity of the inflamed mediastinal glands with glands distant from the site of inflammation. For this purpose the mesenteric glands were suitable. In the sinuses of these glands are often found a considerable number of large mononuclear cells resembling those found in the inflamed gland. In every instance the mesenteric glands were examined microscopically and compared with sections from one or more of the mediastinal glands which were used. The sinuses of the latter were often packed with mononuclear phagocytes in diameter several times that of a polynuclear leucocyte while those of the mesenteric glands contained a moderate number of similar cells only slightly larger than polynuclear leucocytes and very rarely containing ingested material

EXPERIMENT XXIV.—Into each pleural cavity were injected 10 c.c. of aleuronat. At the end of three days when the animal was killed there was no fluid in either cavity. The substernal lymphatic glands were large and succulent and together with several bronchial glands were used to prepare a suspension diluted with salt solution in the proportion of 1: 30. The proteolytic action of 5 c.c. of this suspension and of the same amount of a similarly prepared suspension from the mesenteric glands upon 5 c.c. of heated serum when kept two days at 37° C. is represented by the following figures:

[39] *Beiträge z. chem. Physiol. u. Path.*, 1902, i, 147.
[40] *Zeit. für physiol. Chem.*, 1901, xxxii, 341, 531.

	Mediastinal	Mesenteric
With 0.2% acetic acid	11.45 c.c.	9.9 c.c.
Without addition	4.9 "	5.2 "
With 0.2% sodium carbonate	5.1 "	5.05 "

Control, 5.05 c.c.

EXPERIMENT XXV.—Suspensions, diluted 1:30, were prepared from the substernal and mesenteric lymphatic glands removed four days after the injection of 5 c.c. of aleuronat into the right pleural cavity of a dog.

	Mediastinal	Mesenteric
With 0.2% acetic acid	11.95 c.c.	8.75 c.c.
With 0.2% sodium carbonate	4.25 "	3.8 "

Control (approximately*), 3.3 c.c.

* The quantity of cells obtainable from the lymphatic glands was insufficient for the preparation of a control flask. The amount of nitrogen in uncoagulable form contained in 5 c.c. of serum with 5 c.c. of a suspension of cells from the pleural exudate was determined. The amount of such nitrogen in this quantity of cells is insignificant.

The preceding experiments have shown that suspensions of cells from the inflamed mediastinal glands and from the relatively normal mesenteric glands cause proteolysis in an acid medium but are capable of causing little if any digestion in a neutral or alkaline medium. In both experiments greater proteolysis was caused by the inflamed than by the normal gland. The two experiments which follow confirm this observation.

EXPERIMENT XXVI.—The proteolytic activity of the cells from the mediastinal glands of a dog killed (A) nineteen hours after the injection of aleuronat was compared with that of cells from a dog allowed to live (B) five days after injection. From the latter animal a similarly prepared suspension (1:30) was obtained from the mesenteric lymphatic glands.

	Mediastinal	Mesenteric
A—with 0.2% acetic acid	7.6 c.c.	
B— " " " "	12.2 "	9.c.c.

Control, A (approximately), 2.9 c.c.; B (approximately), 2.9 c.c.

In the following experiment an inflammatory reaction was caused by the injection of red blood corpuscles of the rabbit into the pleural cavities of the dog. The resulting histological changes in the mediastinal lymphatic glands do not differ materially from those produced by aleuronat. The sinuses contain in great number large mononuclear phagocytes actively engaged in ingesting polynuclear leucocytes and red blood corpuscles.

EXPERIMENT XXVII.—A suspension of cells obtained from the mediastinal lymphatic glands of the dog used in Experiment XXIII was diluted with salt solution in the proportion of 1:40 and compared with a similarly prepared suspension from the mesenteric glands. The result of digestion of heated serum during five days at 37° C. was as follows:

	Mediastinal	Mesenteric
With 0.2% acetic acid	7.85 c.c.	5.85 c.c.

Control (approximately), 1.9 c.c.

The foregoing experiments have shown that the proteolytic action of lymphatic glands containing large mononuclear phagocytes, which do not differ from those present in the exudate during the late stages of inflammation, is exhibited in the presence of acid and is almost wholly absent in a neutral or alkaline medium. In the dried powder prepared from washed leucocytes a proteolytic enzyme digesting in a neutral or alkaline medium was found almost wholly free from activity in the presence of acid. It has been possible therefore to obtain separately each of two enzymes demonstrable in the phagocytic cells which have a part in the inflammatory process.

As the length of time after injection of the inflammatory irritant increases mononuclear phagocytes increase in number and size and the exuded cells exhibit an increased ability to digest proteids in the presence of acid. In the neighboring lymphatic glands where cells of similar character accumulate there is an increased proteolytic activity in the presence of acid. The progressively increasing activity of similarly prepared suspensions from mediastinal glands at various intervals after the injection of aleuronat is indicated by the following figures obtained from Experiments XXV and XXVI (in which the conditions are the same) by subtracting the figure representing the control determination of nitrogen in uncoagulable form from that found at the end of digestion.

	1 day	4 days	5 days
With 0.2% acetic acid	4.7 c.c.	8.65 c.c.	9.3 c.c.

When it is recalled that the rapidity of digestion does not vary directly with the amount of ferment but is more closely related to the square root of this quantity the significance of these figures is increased: an increase in the number of mononuclear

phagocytes is accompanied by an increase in the amount of that enzyme which digests proteid in the presence of acid.

The Occurrence of Two Proteolytic Enzymes in the Cells of an Inflammatory Exudate and their Relation to Bone Marrow and Lymphoid Tissue.—It has not been the purpose of the present study to determine the nature of the enzymes which have been found in the leucocytes of inflammatory exudates nor their relation to other somewhat similar bodies. The resistance of both enzymes to a temperature of 70° C. indicates that they are not identical with the alexine or complement, which is destroyed at approximately 56° C. Although the existence of two enzymes has been established, the one present in the polynuclear leucocytes, the other, in the large mononuclear phagocytes or macrophages, there is no evidence that they are identical with the bactericidal complement or microcytase and the hæmolytic complement or macrocytase which Metschnikoff thinks have their origin in these two types of cell. The experiments which have been described have not been concerned with the varieties of the complement nor with its relation to the leucocytes.

That enzyme of which the activity is limited to an acid medium resembles in this respect pepsin, while that which digests in an alkaline medium is more closely related to trypsin. On the one hand the ferment which limits its action to an acid medium is destroyed by means which are used to preserve pepsin, namely precipitation and drying with alcohol and ether, and fails to act in that concentration of hydrochloric acid which is most favorable to the action of pepsin, namely 0.2% (see Experiment II., $\frac{1}{25}$ N= 0.15%). On the other hand that enzyme which is contained in the polynuclear leucocytes and digests in an alkaline medium is apparently far less active than trypsin, obtained from the pancreas by similar methods.

It is not improbable that the enzyme of the mononuclear phagocytes is closely related to if not identical with the autolytic enzyme which is contained in various parenchymatous organs and acts more efficiently in an acid than in an alkaline medium. Repeated experiment has shown that the cells of an apparently normal lymphatic gland are capable not only of autolysis but

of digesting foreign proteid (heated serum) in the presence of acid. Of especial significance, however, is the fact that the amount of enzyme increases when the gland contains an increased number of mononuclear cells capable of intracellular proteolysis.

Recent observers [41] who have studied the morphology and histogenesis of the various types of leucocyte found in the blood with few exceptions support the view long maintained by Ehrlich [42] that the lymphocytes and granular leucocytes differ in origin, one type having its origin in the lymphatic glands and other lymphatic structures, the other in the bone marrow. Concerning the nature of the large mononuclear leucocytes there has been greater divergence of opinion though most observers maintain with Ehrlich and Lazarus that they are closely related to the lymphocytes and have their origin in great part at least in lymphoid tissue. Marchand [43] for example believes that the large mononuclear phagocytes which accumulate in the peritoneal cavity after the injection of various irritant substances are derived from large mononuclear cells situated in the adventitia of the small blood vessels of the omentum and other structures. These cells he designates leucocytoid since they resemble similar cells found within the blood vessels and by division produce not only large phagocytes but he thinks smaller cells which do not differ from lymphocytes. Maximow [44] maintains that the large phagocytes which appear during the course of an inflammatory reaction are derived from lymphocytes which migrate from the blood vessels and has brought forward much experimental evidence in favor of this view. He believes that the adventitial cells of Marchand are of the same nature. Helly,[45] studying the exudates produced by the injection of bacteria into the peritoneal cavity, has reached conclusions similar to those of Maximow.

Corresponding with the two types of phagocyte, one the

[41] See Sternberg, *Lubarsch-Ostertag's Ergebnisse der allg. Path.*, 1903, ix., Abt. ii, 360.

[42] Ehrlich and Lazarus, Normale und pathologische Histologie des Blutes, Nothnagel's spec. Path. u. Ther., 1900, Bd. viii, Tl. i.

[43] *Verhandl. der deutsch. path. Gesellsch.*, 1898, i, 63, and 1901, iv, 124.

[44] *Ziegler's Beiträge*, 1902, Suppl. Hft. v.

[45] *Ziegler's Beiträge*, 1905, xxxvii, 171.

polynuclear leucocyte with fine granulation, the other the large non-granular cell derived from lymphoid tissue, there are two enzymes characterized by their ability to digest proteid, the one in a neutral or alkaline medium, the other in an acid medium. I have shown that the enzyme which is active in the presence of alkali occurs in the bone marrow and that the bone marrow, unlike the liver, spleen, kidney, and other organs, digests proteid more actively in an alkaline than in an acid medium. Since the polynuclear leucocytes with fine granulation—the neutrophile leucocytes of man—are formed in the bone marrow this enzyme doubtless has the same origin and may be designated *leucoprotease.* The name *lympho-protease* may be given to that enzyme which is contained in the large mononuclear phagocytes since these cells have their origin in lymphoid tissue.

THE TOXICOLOGICAL CONSTITUTION OF AMANITA PHALLOIDES.

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It has been pointed out in previous papers[1] that the most deadly of all poisonous fungi, and the one responsible for the majority of deaths from fungus eating, is Amanita phalloides, the hæmolytic principle of which was discovered in 1891 by Kobert,[2] who gave it the name phallin. This hæmolysin, in virtue of its acting directly upon the red blood corpuscles without the intermediation of serum, its inactivation at 65° C., and its failure when inactivated to be reactivated by substances containing complement for serum hæmolysins, should be classed provisionally with the bacterial hæmolysins.

It has also been shown that extracts of Amanita phalloides are profoundly poisonous to animals, the latter showing lesions characteristic of bacterial intoxication in general, and that non-lethal doses are capable of causing an immunity in susceptible species. The serum from immunized animals is antihæmolytic and antitoxic in character, a minimal hæmolytic quantity for one cubic centimeter of a 5 per cent. blood suspension being neutralized by certain sera in a dilution of 1:10,000, and ten times a fatal dose for rabbits being neutralized by one cubic centimeter of the same sera. Such sera can be compared with the old and weaker normal antidiphtheritic sera where one cubic centimeter neutralized ten times a fatal dose of the diphtheria toxin for a guinea-pig of 250 grams. It is now necessary to consider in greater detail the lesions produced in animals by this poison, and to determine what relation these bear to the blood-laking properties of Amanita phalloides.

According to Kobert's original publications, there is but one

[1] Ford, *Medical News*, 1905, lxxxvii, 771. *The Journal of Infectious Diseases*, 1906, iii., 191.

[2] Kobert, *St. Petersburger medicinische Wochenschrift*, 1891, xvi, 463; 471.

poisonous substance in this fungus, phallin, and the changes seen in fatal intoxication in man and the lesions produced experimentally in animals by it are due entirely to blood destruction. Kobert regards phallin as a toxalbumen in virtue of its thermolability, and intravenous injections cause intravascular dissolution of the corpuscles. The specimen employed by him was of enormous hæmolytic strength, ox corpuscles being dissolved by the dried extract, in a dilution of 1:125,000.

For the purposes of our present study, the lesions produced in experimental animals may be briefly stated to be the following[3]:

I. Subcutaneous gelatinous œdema;
II. Hæmorrhagic lymphatic glands;
III. Hæmoglobinuria;
IV. Hæmorrhages in serous membranes and parenchymatous organs;
V. Necrosis and fatty degeneration of heart muscle, liver, and kidney;
VI. Pigmentation of the spleen.

The pigmentation of the spleen and the hæmoglobinuria can be referred at once to the hæmolysis produced by extracts of Amanita phalloides, since these changes are characteristic of hæmolytic intoxications in general. Can we with equal confidence, however, consider the other changes, namely, the œdema, hæmorrhages, and necrosis, as due to hæmolysis?

If we inoculate a considerable series of animals, the lesions described are seen with great regularity. There is, however, a vast difference in the amount of œdema, the extent and distribution of hæmorrhages, and the degree of tissue degeneration. As we approach the lower limits of a fatal dose, the œdema at the site of inoculation becomes proportionately less, the hæmorrhages may be lacking altogether or at least the evidence of them at autopsy is wanting, and the extent of the tissue change, especially the fatty degeneration of the liver, becomes greater and greater. This variation in the character of the lesions suggests that the disturbances produced are greater than can be explained by blood

[3] The lesions produced by Amanita phalloides will be more fully described in another paper.

destruction alone. If guinea-pigs, which are very susceptible to the toxic effects of this Amanita, be inoculated with minimal amounts of the poison they will frequently die in a short time and fail to present, at the site of inoculation, either œdema or hæmorrhage; and upon microscopical examination of the organs no pronounced lesions will be found. Nevertheless these animals develop much the same toxic symptoms as other animals which have received a larger fatal dose.

If we estimate the amount of blood destruction produced by these small lethal doses of the Amanita, an unexpected fact is brought out: the degree of blood destruction is not proportional to the profundity of intoxication. A few examples will be given.

A guinea-pig of 240 grams weight succumbed quickly to a quantity of the Amanita which outside the body is capable of dissolving about one cubic centimeter of undiluted blood; and a rabbit of 870 grams weight died in forty-eight hours from a dose of the poison which dissolves only $2\frac{1}{2}$ cubic centimeters of rabbit's blood. The loss of such small quantities of blood can be borne easily by animals of these species. In estimating the hæmolytic strength of doses of the extract capable of producing either rapid or slower fatal results, the same lack of proportion between the amount of blood destroyed and the severity of the lesions was apparent.

In endeavoring to explain the facts just stated several possibilities present themselves. The toxic effects produced by the Amanita may be secondary, in that the intravascular alteration of blood corpuscles may cause changes associated with thrombosis and infarction, to which the subsequent necrosis and tissue destruction are added; or the hæmolytic principle may be less specific than is supposed and exert an injurious effect upon other cells of the body; or finally, still other poisons in addition to the one discovered by Kobert may exist in the Amanita.

As regards the first of these considerations, it may be stated that Amanita phalloides does not agglutinate blood corpuscles *in vitro*, or, so far as can be concluded from a microscopic examination of the tissue, *intra vitam*. The tissue degeneration cannot, therefore, be explained by thrombi of agglutinated erythrocytes

as some of the lesions produced by ricin and abrin have been accounted for. It is also no more easy to explain the lesions on the basis of simple destruction of a small number of red blood cells.

The second and third of these considerations can be tested experimentally. It has been pointed out that extracts of Amanita phalloides lose their hæmolytic activity entirely when heated to temperatures between 65° and 80° C., and no substance has been discovered capable of reactivating them. This inactivation may, therefore, for our present purpose be considered as destruction. Animals inoculated with extracts heated to various temperatures can be used to determine whether the entire toxicity disappears as the hæmolysins are rendered inactive. A series of rabbits and guinea-pigs was inoculated with different quantities of the Amanita extracts previously heated for one half hour to 60°, 65°, 70°, 80°, 90°, and 100° C. All the animals died after brief periods except those given the extract heated to 90° and 100° C. The extract heated to 60° C, caused, except for slighter subcutaneous œdema, lesions identical with those produced by the raw material. Hence a temperature of 60° C. fails to exert any special action upon the Amanita poison. The extract heated to 65°, to 70°, and to 80° C. is still toxic, killing animals almost as rapidly as the unheated material, but the pathological changes produced are greatly modified. Subcutaneous œdema and hæmoglobinuria are now absent; hæmorrhages are still present, but to a less degree, and the necrosis and fatty degeneration of the parenchymatous organs are diminished. Upon microscopic examination the pigmentation of the spleen is seen to be lacking, but all the other changes occur and are identical with those produced by the crude extract. With the loss of hæmolytic activity the extract has lost its power of producing subcutaneous œdema, hæmoglobinuria, and pigmentation of the spleen. The toxins are completely destroyed at a temperature between 90° and 100° C. The following protocols give the results of the heating experiments.

EXPERIMENT I. Rabbit, 1200 grams, inoculated February 18, 1905, with 1 c.c. of an extract of Amanita phalloides heated to 60° C. for ½ hour. Death Feb. 20. Autopsy: Subcutaneous œdema, hæmorrhages, and necrosis.

EXPERIMENT II. Rabbit, 1270 grams, inoculated Feb. 13, 1905, with the same, heated to 70° C. for ½ hour. Death Feb. 14. Autopsy: No exudate at site of inoculation. Hæmorrhages in lymphatic glands and adrenals. Liver congested; areas of hæmorrhage on its surface. Spleen dark and soft, bladder filled with normal looking urine. Hæmorrhagic areas in one lobe of the lung; a few minute hæmorrhages in the mucosa of the intestines. Cultures sterile.

EXPERIMENT III. Rabbit, 1445 grams, inoculated Feb. 13, 1905, with 1.2 c.c. of the same extract heated to 80° C. for ½ hour. Death Feb. 14. Autopsy: Slight congestion at site of inoculation, but no œdema. Lymphatic glands everywhere hæmorrhagic. Liver congested, but without definite areas of hæmorrhage. Spleen large, dark, and soft. Kidneys and adrenals show no definite hæmorrhages. Bladder filled with pale urine. Heart dilated; blood in heart and larger vessels clotted. Lungs, stomach, and intestines, negative. Cultures from the organs sterile.

EXPERIMENT IV. Rabbit, 1230 grams, inoculated Feb. 13, 1905, with 1 c.c. of the same extract heated to 90° C. for ½ hour. No symptoms; survived.

EXPERIMENT V. Rabbit, 1400 grams, inoculated Feb. 13, 1905, with 1.2 c.c of the same extract boiled for ½ hour. No effects.

Similar experiments which were carried out upon guinea-pigs gave the same definite results. The outcome of all these experiments shows that the extracts, when heated to 65° and 80° C., retain their toxicity, and cause death within forty-eight hours, but the lesions have entirely changed in character. In guinea-pigs a little œdema sometimes develops at the site of inoculation, but this is not of the same character as that produced by the unheated material. It probably merely indicates a great susceptibility of the blood-vessels to the poison, just as the corpuscles of the guinea-pig are so susceptible to the action of the Amanita that at times a very few corpuscles will be destroyed by the extract heated to 65° C. and even to 70° C.

Extracts of Amanita phalloides which have completely lost their hæmolytic activity still retain their toxicity. Hence after depriving these extracts of their phallin by heat there must still remain certain substances which are capable of causing rapid death, and of setting up those changes in the animal body associated with hemorrhage, necrosis, and fatty degeneration of the parenchymatous organs. To destroy completely the Amanita, temperatures of 90° to 100° C. are required. Hence I conclude that extracts of the fungus contain thermolabile and thermostable substances.

Amanita Extracts Treated with Blood.—Still another method

for determining the toxic constitution of the Amanita is to treat the extracts with various cells of the body to ascertain whether they can thus be neutralized. If the extracts owe their toxicity primarily to a blood-laking poison, then the complete saturation of this poison by blood ought theoretically to give us a neutral mixture.

A number of combinations of corpuscles and the extracts were made for this purpose and the results were uniformly negative. Thus three cubic centimeters of an extract were mixed with twelve and a half cubic centimeters of washed rabbit corpuscles; hæmolysis occurred in about twenty minutes. This proportion of blood corpuscles and extract should theoretically give a neutral mixture, assuming the poison to be primarily a hæmolytic one. About eight cubic centimeters of this mixture, representing more than a fatal dose for certain animals, were given to a rabbit. Death took place after seven days and the post-mortem appearances were those of the Amanita intoxication.

If the extracts consisted chiefly of a blood poison whose activity is inhibited but not destroyed at 80° C., then the heated material should be capable of neutralization and be rendered non-toxic by saturation with blood corpuscles. Here also the experimental results were negative. Thus three cubic centimeters of an extract were combined with twelve and a half cubic centimeters of washed rabbit blood corpuscles, and eight cubic centimeters of this mixture were given to a rabbit. Such a mixture should theoretically be neutral. The inoculated animal, however, died at the end of twenty-four hours, and showed characteristic appearances of Amanita poisoning.

From these experiments it can be concluded that blood corpuscles are in themselves unable to neutralize Amanita extracts, and that the poisons contained in this fungus are not exclusively active against these cells.

Extracts Treated with Brain Tissue.—Many of the symptoms of Amanita intoxication point to a direct action of the fungus upon the nervous tissues. The experiments of Wassermann[4] upon the neutralization of tetanus toxin by emulsions of brain tissue

[4] Wassermann and Takaki, *Berliner klinische Wochenschrift*, 1898, xxv, 5.

and those of Flexner and Noguchi[5] upon the neurotoxic constituent of cobra venom have suggested another mode of analysis of the toxic constituents of this poison. If the chief poisons of the Amanita are neurotoxic, the blood-laking constituent being an ingredient of secondary importance, the combination of the Amanita extract with brain tissue should give a mixture acting still on the blood but without action on the nerve centres. Hence animals receiving this treated poison should recover, as the blood destruction is too small to cause, by itself, fatal intoxication. The results of this series of experiments are given in the following protocols:

EXPERIMENT I. The brain of a rabbit, excised aseptically, was macerated in salt solution and mixed with 5 c.c. of Amanita extract. After several hours' contact a guinea-pig of about 300 grams weight was inoculated with 1 c.c. of the supernatant fluid. Result: death in 18 hours; usual autopsy findings.

EXPERIMENT II. The brain of a guinea-pig, excised aseptically, was macerated in salt solution and mixed with 2 c.c. of a similar extract. The mixture was kept three hours at room temperature and then injected subcutaneously into a guinea-pig of 650 grams. Death in 18 hours.

EXPERIMENT III. A whole brain of a guinea-pig, excised aseptically, was macerated in salt solution and mixed with 4 c.c. of the extract. Two and one-half c.c. of this emulsion were given to a guinea-pig of 400 grams weight Death in 24 hours.

From these experiments, it may be concluded that brain tissue has not the power to remove or neutralize the poisonous principles of Amanita phalloides.

Since, however, the experiments with heat indicate that the thermolabile substance is definitely hæmolytic, it may be that the thermostable substance is neurotoxic in character, and hence the effect of the inoculation of the crude material depends upon the presence of these two poisons. It was desirable, therefore, to destroy the hæmolytic principle by heat and to extract the heated poison with brain tissue in order to determine whether the thermostable substance is really a neurotoxin. The toxicity could not be removed in this manner as the following experiment shows:

[5] Flexner and Noguchi, *University of Pennsylvania Medical Bulletin*, 1902–03, XV, 345.

An extract of the Amanita was heated to 80° C. to destroy completely the hæmolysin, and 2 c.c. were mixed with the brain of a guinea-pig, which had been removed aseptically and emulsified in salt solution. The entire mixture was injected into a 400-gram guinea-pig, which died in two weeks and exhibited changes characteristic of Amanita poisoning.

I therefore conclude that the fungus does not contain a neurotoxin capable of being neutralized by brain tissue.

Effect of Artificial Digestion upon Extracts of the Amanita.—The fungus having been shown to contain two distinct principles—one thermolabile and the other more heat resistant—it is of interest to ascertain whether they show any differences to the action of ferments. To test this question the crude extracts, before and after heating, were digested for a number of days with pepsin and pancreatin and their hæmolytic strength and general toxicity ascertained. Table I shows that the unheated extract employed caused complete hæmolysis of 1 c.c. of a 5 per cent. suspension of rabbit corpuscles in quantities at and above 0.003 c.c., while 0.001 c.c. produced a trace of hæmolysis.

TABLE I.

Crude extract of Amanita.	5% rabbit blood.	Hæmolysis.
1.0 c.c.	1 c.c.	Complete
0.1 "	" "	"
0.05 "	" "	"
0.01 "	" "	"
0.005 "	" "	"
0.003 "	" "	"
0.001 "	" "	Trace
Control 1 c.c. Na Cl 1%	" "	None

This experiment was repeated separately with pepsin and pancreatin. In each case the crude extract was digested for eight days with pepsin and with pancreatin, and in both the hæmolysin was entirely destroyed, since when mixed in equal quantities—one cubic centimeter of the extract and one cubic centimeter of the blood suspension—no hæmolysis occurred.

The influence of the ferments on the thermostable poison was either wholly negative, or, at best, very slight.

EXPERIMENT I. Guinea-pig weighing 670 grams. Injected subcutaneously 2 c.c. extract heated to 65° C. for ½ hour and digested with pepsin for 7 days. Death in 24 hours. The autopsy showed the characteristic lesions of the heated extract including extensive hæmorrhage at the site of inoculation.

EXPERIMENT II. Guinea-pig, 440 grams, inoculated with 1 c.c. of the extract heated to 65° C. and digested with pancreatin. Dead in 24 hours. Autopsy revealed usual changes.

EXPERIMENT III. Guinea-pig, 447 grams, inoculated subcutaneously with 1 c.c. extract heated to 80° C. for ½ hour and digested with pepsin for 7 days. Dead in 24 hours. Autopsy: Hæmorrhage at site of inoculation, and dilated heart.

EXPERIMENT IV. Guinea-pig, 395 grams, inoculated with extract heated to 80° C. and then digested with pancreatin for 7 days. Death followed inoculation within a very short time, possibly less than 1 hour. The material given to this animal, owing to its evaporation, represented a considerable multiple of the fatal dose.

These experiments show that the toxic substance in the Amanita which resists heat also resists the action of pepsin and pancreatin. That the crude extract is, moreover, still toxic after artificial digestion for two weeks, is shown by the fact that a dose of two cubic centimeters killed a guinea-pig weighing 420 grams in four days.

Constitution of the Amanita.—From these experiments dealing with the action of heat and of ferments upon extracts of the Amanita, and from the attempt to neutralize this substance with blood and nerve tissue, and, finally, from a consideration of the post-mortem changes, we obtain a different idea of the constitution of the fungus from the one held by Kobert. While the hæmolytic ingredient, the phallin of Kobert, is an important constituent of the fungus and exerts a very powerful action upon all erythrocytes, it is by no means the only substance present which exerts a toxic effect on animals. In addition to phallin, which is thermolabile and susceptible to artificial digestion, there is present a powerful thermostable substance resistant to the action of ferments. To the hæmolysin are to be ascribed the subcutaneous œdema, hæmoglobinuria, and pigmentation of the spleen produced in artificial intoxication; while to the action of the thermostable substance, which is non-hæmolytic, are due the hæmorrhages, the necrosis, and fatty degeneration in various organs. Whether the hæmolytic substance can produce necrosis, fatty degeneration, and hæmorrhage cannot at the present time be stated definitely, since the different ingredients of Amanita

have been separated by chemical means, the thermolabile substance having been obtained free from the thermostable body, only within the past few weeks. It is hardly conceivable, however, that a substance acting so energetically upon blood corpuscles should not also exert a necrotizing action upon other cells of the body. The thermostable body is the most important constituent of Amanita and the one causing the most profound disturbance of the organs and tissues.

It has been pointed out in a previous paper that by the immunization of animals it is possible to obtain antibodies to extracts of Amanita phalloides which are both antihæmolytic and antitoxic in character. In view of the observations outlined in this paper the question may be asked whether this antiserum is a single body capable of neutralizing both the hæmolytic and the toxic action of the Amanita, or whether it is a combination of antibodies, each acting upon the especial substance to which the animal is immunized. To put the question in another way, it may be asked whether the particular chemical groups under whose influence the antibodies for the Amanita are produced, are identical in the thermolabile and the thermostable substances, or are quite different. If, according to Ehrlich's ideas, it be granted that every toxin is composed of two chemical complexes, one the haptophoric group uniting with susceptible cells, the other the toxophoric group causing the injury of these cells, the haptophoric group alone taking part in the processes leading to artificial immunity and the production of antibodies; then in the case of Amanita the toxophoric group of the hæmolytic or thermolabile constituent must be quite distinct from the toxophoric group of the thermostable constituent; since these groups attack different cells of the body, the one acting primarily upon the erythrocytes, the other primarily upon the cells of the parenchymatous organs. Two possibilities are, however, open in considering the haptophoric groups of these two constituents of the fungus. On the one hand, the haptophoric groups may have the same structure, in which case but one antibody should be found in the antiserum, the antithermolabile substance being identical with the antithermostable substance. On the other

hand, the haptophoric groups may be different and thus the antiserum contain two distinct antibodies, one neutralizing the hæmolysin, the other neutralizing the resistant body.

If the hæmolysin of Amanita is destroyed by heat, and an antibody be made from the residue, and if the thermolabile and the thermostable bodies are identical in haptophoric structure, the antibody produced should be antihæmolytic and at the same time antitoxic. If, on the other hand, the haptophoric groups be different, the antibody produced for the thermostable body should be merely antitoxic and have no antihæmolytic power. Such an experiment should also settle definitely the question whether there are two separate toxic substances in Amanita phalloides, in contradistinction to the notion that the action of heat and of digestion is merely to alter the arrangement of molecules in a toxic complex consisting of a single substance, in which occurs one haptophoric group attached to at least two toxophoric groups presenting differences of resistance.

Jacoby,[6] who has investigated the toxic and agglutinating action of ricin, arrived at the conclusion that this poison contains an agglutinin and a toxin, each of which possesses separate and distinct haptophoric groups. At Ehrlich's suggestion, however, Jacoby immunized a goat to ricin from which all traces of agglutinin had been removed by combination with blood corpuscles, and as the resulting antiserum was anti-agglutinating as well as antitoxic, Jacoby was led to believe that the ricin-toxin and the ricin-agglutinin possessed identical haptophoric groups.

A series of animals was immunized to extracts of Amanita heated respectively to 65° C. and to 80° C. for one half hour. It proved to be somewhat less difficult to immunize the animals to the heated than to the unheated extracts, since the local œdema and necrosis at the site of inoculation were by this means largely avoided. The immunization was pushed to the point where the animals withstood the inoculation of several multiples of the fatal dose of the heated material, after which the blood was withdrawn and tested for its antihæmolytic and its antitoxic properties.

[6] Jacoby, *Hofmeister's Beiträge*, 1902, ii, 535.

A rabbit of 1970 grams was given on successive occasions an extract of Amanita which had been previously heated to 65° C. for one-half hour. The acutely fatal dose was 1.2 cubic centimeters.

Date.	Dosage.
May 5	0.3 c.c.
" 12	0.6 "
" 19	0.9 "
" 26	1.2 "
June 10	1.4 "
" 19	1.75 "
" 28	2.00 "

Bled June 29, 20 c.c.

This serum was tested for its antihæmolytic and antitoxic effects.

TABLE II.

ACTION OF IMMUNE SERUM ON HÆMOLYSIS.

Extract.	5% rabbit blood.		Serum.		Hæmolysis.
.005 c.c.	+ 1 c.c.	+	0	=	Complete
.005 "	+ 1 "	+	0.1 c.c. Normal	=	"
.005 "	+ 1 "	+	0.05 " Immune	=	"
.005 "	+ 1 "	+	0.1 " "	=	"

The immune serum made from the heated extract does not prevent hæmolysis. The next experiments were made in order to test the antitoxic properties of the serum.

Control: 1 c.c. of an extract heated to 65° C. for ½ hour kills a rabbit of 1500 grams in a short time.

EXPERIMENT I. Rabbit, 1050 grams, inoculated July 1 with 1 c.c. of the extract heated to 65° C. for ½ hour + 1.5 c.c. of the immune serum. Animal unaffected.

EXPERIMENT II. Rabbit, 1150 grams, inoculated July 1 with 1 c.c. of the extract heated to 65° C. for ½ hour + 1.5 c.c. of the immune serum. Animal unaffected.

The immune serum, although completely devoid of antihæmolytic action, therefore possesses definite antitoxic properties.

A second rabbit was immunized to the Amanita extract heated to 80° C. for half an hour, and its serum was tested both for antihæmolytic and for antitoxic properties. The dose of the extract which quickly caused death was 1.2 cubic centimeters.

A rabbit weighing 1860 grams was immunized as follows:

Date.	Dosage.
May 5	0.3 c.c.
" 12	0.6 "
" 19	0.9 "
" 26	1.2 "
June 6	1.4 "
" 16	1.7 "
" 26	2.0 "

July 5, 15 c.c. blood withdrawn.

The serum of this animal was wholly devoid of antihæmolytic properties, being without effect in a 1:10 dilution. Its antitoxic action was marked.

EXPERIMENT I. Rabbit, weight 1500 grams, inoculated July 6 with 2 c.c of the extract heated to 80° C. ½ hour + 2 c.c. antiserum. Recovered.

EXPERIMENT II. Rabbit, weight 1500 grams, inoculated July 6 with 2 c.c. of the extract heated to 80° C. for ½ hour + 1 c.c. antiserum. Died.

EXPERIMENT III. Rabbit, weight 1600 grams, inoculated July 6 with 2 c.c. of the extract heated to 80° C. for ½ hour + 0.5 c.c. antiserum. Recovered.

The antiserum possesses, therefore, definite antitoxic properties, two cubic centimeters completely neutralizing an excess of the toxin representing about five times the lethal dose for a rabbit of this weight. Quantities less than two cubic centimeters failed to protect regularly. The antiserum is also effective for guinea-pigs.

Control: I c.c. of the heated extract kills a guinea-pig in 24 hours.

EXPERIMENT I. Guinea-pig inoculated July 6 with 1 c.c. of the extract heated to 80° C. for ½ hour + 1 c.c. antiserum. Recovered.

EXPERIMENT II. Guinea-pig inoculated July 6 with 1 c.c. of the extract heated to 80° C. for ½ hour + ½ c.c. antiserum. Recovered.

EXPERIMENT III. Guinea-pig inoculated July 6 with 1 c.c. of the extract heated to 80° C. for ½ hour + ¼ c.c. antiserum. Recovered.

We thus see that the antiserum produced by the immunization of animals to the thermostable substance contained in the extract of Amanita phalloides heated to 65° and to 80° C. neutralizes the toxic action of this substance only, having no power to neutralize the thermolabile or hæmolytic principle. The two substances must, therefore, possess separate haptophoric and toxophoric groups. They do not, therefore, consist of a complex molecule

composed of a single haptophore group united to at least two toxophore groups.

Since the completion of this paper an investigation of the chemical constituents of Amanita phalloides has been carried on in the laboratory of Professor J. J. Abel, the report of which will appear shortly. The separation of the two principles described has now been accomplished by chemical means.

CONCLUSIONS.

From the experiments detailed in this paper it may be concluded that Amanita phalloides contains besides phallin, or the hæmolytic principle of Kobert, another body of toxic nature. Phallin is thermolabile, and is destroyed by the action of pepsin and pancreatin. The other toxic body is thermostable and is resistant to pepsin and pancreatin. The two substances, moreover, possess different toxophoric and haptophoric groups, since an antiserum produced by the immunization of animals to the thermostable body has no neutralizing effects upon phallin.

The thermolable body, phallin, produces the subcutaneous œdema and hæmoglobinuria, and, in virtue of its blood-laking properties, the pigmentation of the spleen. The thermostable body produces hæmorrhage and necrosis, and the fatty degeneration of the parenchymatous organs. The two bodies exist side by side in watery extracts of the fungus, but they cannot be considered as two constituents of a single poison exerting a variety of effects. To the hæmolysin the name phallin has already been given by its discoverer, Kobert. For the thermostable substance described now for the first time, the name Amanito-toxin is proposed provisionally.

CONCERNING THE MORPHOLOGY AND REPRODUCTION OF SPIROCHÆTA PALLIDA AND A RAPID METHOD FOR STAINING THE ORGANISM.

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PLATES XXI AND XXII.

The object of this paper is to describe a simple method for the staining in smear preparations of Spirochæta pallida and to present certain observations which I have been enabled to make upon the morphology and reproduction of the micro-organism through the use of this staining method. I have examined a large number of cases of syphilis and various syphilitic lesions, and it is upon the study of a relatively large mass of material that I base my conclusions. I have purposely omitted references to the literature of my subject as this communication is intended to be a report of personal observations.

The Giemsa staining method, which is based upon the Nocht-Romanowsky principle of staining, is so time-consuming, where the question of a possible quick differential diagnosis is to be considered, as to be quite useless. Besides this, we have to deal with the disadvantage that the organism is stained so lightly as at times to be readily overlooked. One can safely state that there is no modification of the Romanowsky method which will not stain the pallida, but I believe the results to be less brilliant than those obtainable by the method I am about to describe.

I prepare a dye for Spirochæta pallida as follows:

In 200 cubic centimeters of water two grams of lithium carbonate are dissolved and two grams of methylene blue are added. While some other carbonate may be used, the use of lithium salt seems to me preferable. The methylene blue is either Merck's medicinal, or Gruebler's B X, or Koch's rectified. This alkaline methylene blue solution is heated in a rice-boiler with a moderate amount of heat until a rich polychrome has formed. The steps

leading up to this formation are followed by taking every few minutes a sample in a test-tube, and examining by holding it against artificial light. As soon as a distinctly red color is obtained the desired degree of heating has been reached. During the cooling, which is allowed to take place spontaneously, more of the polychrome will be formed. The residue which is undissolved is removed from the cooled dye by pouring through cotton in a funnel.

To one half of this alkaline polychrome dye 5 per cent. acetic acid is gradually added until a strip of litmus paper shows above the line of discoloration a distinct acid reaction. The remaining half of the dye is now added, so as to carry the reaction back to a low degree of alkalinity.

A weak eosin solution is now prepared, approximately one half per cent. French eosin, and this is added gradually, while the mixture is being stirred, until a filtered sample shows the filtrate to be of a pale bluish color with slight fluorescence. The mixture is allowed to stand for one day and then filtered. The precipitate which has separated is collected on a double piece of filter paper and dried at room temperature, or at a temperature not exceeding 40° C. Excessive heat injures the dye.

When this precipitate is completely dried, it is readily removed from the filter paper and may then be dissolved without further washing in commercial wood alcohol.[1] The solution should be allowed to stand for a day in an open vessel, and then filtered. An alcohol insoluble residue may be present which is to be removed. The strength of this alcoholic solution is approximately 1 per cent.

To use the stain on slide smears, one drops sufficient dye on an unfixed preparation to cover it, allows the dye to act for three or four seconds, and pours off the excess of dye. The slide is now introduced *slowly* into clean water with the *film side down*, permitting in this manner an interaction between the film of adhering dye and the water. The slide is held in this slanting position in the water for another four or five seconds, and is

[1] Commercial wood alcohol is to be preferred to the pure article, as it gives better fixations.

next shaken in the water so as to wash off the excess of dye.

The reason for holding the preparation stained side downward is to prevent the forming precipitate from falling upon the smear and disfiguring it. A well-stained preparation is obtained in this manner, and the pallida appears of a violet color, which may be changed to bluish-black by flooding the preparation for fifteen or twenty seconds with the ordinary iodo-iodide of potash solution of Gram, washing and drying as usual. This counterstaining with Gram's solution permits a moderate degree of differentiation with alcohol, but this has up to the present offered no advantages, as the pallida decolorizes readily.

My study has embraced sixty cases of undoubted syphilis, and of these only eight have failed to show the pallida. The results depend very much upon the technique employed. Repeated examinations were not infrequently necessary before the parasite could be demonstrated. Moreover, as it could not be demonstrated at all times in every lesion of the same individual, the desirability of making preparations from both primary and secondary lesions located in different parts of the body is evident. The number of pallida found did not seem at any time to bear relation to the severity of the infection. The mere fact that numerous organisms could be obtained one day from a lesion, while on the following day but few were obtained from the same lesion, although the same technique was employed, goes to show that numbers are not always the most important factor in determining success of demonstration. The appearance and grouping of the pallida frequently suggests other possibilities, as I will show presently.

Since I lay much stress upon technique, I will describe the method with which I obtained my best results: curettage was always resorted to with a view to removing surface epithelium and exudate, the latter especially, because the finding of the pallida in smears showing pus proved to be a matter of great difficulty. The curettage was carried sufficiently far to cause the appearance of a small amount of serum and blood, and it was preferably done at the edge of the lesion rather than in the

center. The deeper curettages also helped to eliminate other organisms from the field. Wherever it was possible, the making of impression preparations was preferred, not only because of the ease with which they are made, but because they proved to be of advantage in the study of the morphology and the probable mode of reproduction of the pallida. A lesion of the throat had to be handled differently. Gentle curettage usually caused the appearance of a little blood which was removed with a sterile platinum wire and smeared upon a slide; in fact the majority of mouth lesions and those located on the vaginal wall had to be studied in this manner. In the examination of roseola efflorescences the curettage was steadily carried to the bleeding point in half a dozen or more places, and a number of preparations were made, some being taken at the moment preceding the appearance of blood corpuscles, because it was found that the optimum point was readily overstepped when it was a question of finding the spirochætæ rapidly. Under all circumstances the finding of the parasite in these roseolæ was more or less difficult.

Blood smears were frequently taken from the lobe of the ear in the usual manner and examined carefully, especially in those cases where a chancre was of short standing, but this tedious work remained entirely negative as far as finding the organism was concerned. Moist papules seemed to contain the largest numbers, and incidentally it may be stated that in many instances the longest individuals were found in them. The majority of chancres showed almost equal numbers, but only after the curettage had been carried out at the edge of the lesion.

Several cases studied were of exceptional interest. I will state briefly some of the most important features of them.

Case 1.—Female who denied all knowledge of a primary lesion. Body covered with a papulo-macular eruption. On careful examination a small moist papule was found concealed under the left mamma. Slides were prepared from the moist papule and from the eruption on the arm and shoulder. Impression preparations from the moist papule showed the pallida in enormous numbers, many fields showing from twenty to thirty individuals. The macules from the arm and shoulder showed thousands of

spirochætæ, but among them pallida forms were not discovered. The types seen were refringens, and other straighter forms.

Case 2.—Male. Papules on scrotum and penis. Macules, general. The former showed after repeated examination a small number of pallida, the latter failed to show pallida. The papules were of four weeks standing.

Case 3.—Female. Examination extending over eight weeks during administration of mercury. Mucous placques were studied. The pallida was found at each examination, but the last two examinations showed diminution in numbers. In this connection another case may be mentioned in which the examination of mucous patches gave on two occasions positive and in the intermediate examination negative results.

Several lesions which were negative were as follows:

Mixed (?) lesion of the penis; double primary lesion of the tongue and penis (uncertain history of infection); tubercular syphilide over the right eye. The last showed the refringens.

Late lesions, believed to be tertiary, were repeatedly examined with negative results.

Control examinations of non-syphilitic lesions were also made. In a case of acne and another of eczema spiral organisms were found, but they could be distinguished from the pallida. They were thicker and the turns less sharp; ends pointed. Smegma gave some suggestive organisms: they were as delicate as pallida but the characteristic sharp turns of the latter were absent. These last organisms when suspended in salt solution and kept at body temperature were highly motile.

In morphology, the pallida which I studied agreed, in general, with the description of Schaudinn and Hoffmann. As regards the question of flagella, I found on occasional organisms what might have been considered such appendages. I examined hundreds of spirochætæ in impression preparations in order to find one organism showing the prolongation. It seemed indeed too thick to be an ordinary flagellum. I was unsuccessful with Loeffler's stain. In the fresh state I could not discern flagella.

The length of and hence the number of curves in the pallida varied greatly. As many as twenty-nine curves have been

counted (Plate XXII, Fig. 10), on an individual of uniform thickness. In thickness there is also variation, so that the dimensions given by Schaudinn and Hoffmann do not hold absolutely. (Plate XXI, Fig. 4, a.) The thickness may approach that of refringens; and in a chancre of the lip I found two pallida of unusual length. One possessed forty turns at least, and stretched across the greater part of the microscopic field. It was very thin and faint. I regard the pallida as a spiral of variable length and thickness, attenuated at the ends so as to form a fine point, the terminal end being as a rule no more than one coil in length.

Impression preparations which were dried immediately showed not infrequently organisms of the pallida type which, in places, were rather straight, so that I do not believe the spirochæta to be a rigid spiral; but, on the contrary, I believe it to be very flexible. The living organism presents rather constantly the spiral form, if the medium in which it moves be sufficiently abundant and fluid. I have made it a rule to place a thin hair between the cover-glass and the slide, so that close proximity of the cover may not interfere with the movements of the parasite, and to allow the fluid to enter by capillarity. I prefer such preparations to the hanging drop. The movements of the pallida may be compared to those of the spirochæta of relapsing fever, except that those of the pallida are slower even at room temperature. The pallida seems to move with equal facility slowly backward and forward. It seems to turn on its axis, and while its movements are evident, it appears to remain rather stationary.

The impression preparations seemed at first to yield information regarding the mode of reproduction of the pallida. At the February meeting of the New York Pathological Society I expressed the belief that longitudinal division occurred, leading to the production of three or four individuals which remained attached end to end, before separation by transverse section. On Plate XXII Figure 4, e, shows, for example, a relatively thick, short parasite which becomes attenuated at one end, from which a second organism seems to arise and split off. Still other organisms are short and thick and give rise, apparently, at one end to several individuals which can be made out dis-

tinctly. Moreover, I was able to show in a single field of the microscope ($\frac{1}{12}$ inch immersion lens) as many as five spirochætæ, which on close examination proved to be double, and still other spirals seemed to be uncoiling (Plate XXII, Figure 9). These appearances I at first interpreted as examples of longitudinal division.

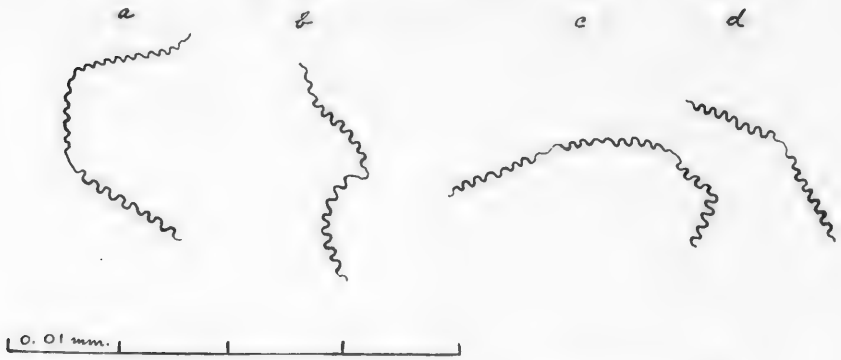
I have now come to the conclusion that the intertwining can be accounted for in a simple way by considering the effects of (1) the mode of progression of the organisms, (2) the large numbers which are present in the lesions, and (3) the consistence of the medium in which they move. Of these factors the first one is doubtless the most important.

In order to obtain a better understanding of the appearances suggesting longitudinal division, I prepared two wire models, coiled as the pallida is supposed to be. By carelessly throwing them together a double spiral, permitting of lateral separation, was eventually formed. By rotating one spiral to the right and the other to the left, one lying behind the other, all figures from incomplete to complete apposition, with the production of a thickened spiral, were reproduced. In this condition lateral separation, when one remembers the flexibility of the pallida, is seen to be possible, especially if the individuals moved in opposite directions. What, however, is unaccounted for and, by this view, impossible to account for, is the end to end attachment forming a continuous and long spiral organism with attenuated points in two or three places (Plate XXI, Figure 2).

There was another objection to the view of longitudinal division: the number of turns in the apposed organisms was subject to too great variation. While one organism showed six turns, the other showed as many as ten. This appearance could be more easily explained on the supposition of entanglement.

Not infrequently I noticed spirals of the pallida type which were twice the length of typical specimens. Near the middle a thinned out, more or less straight part appeared, and beyond, on each side, the curves reappeared equally. To account for this appearance as the result of longitudinal division, one might suppose the individuals to have whipped apart, while the two ends remained

united. This was the attenuated section. In endeavoring to account for these different appearances on the basis of longitudinal division, I did not leave out of account the possibility of



Transverse division of Spirochæta pallida. Camera lucida drawings.
a. Organism dividing to form probably three; curves and diameter irregular.
b. Characteristic division.
c. Division into three; organisms irregular.
d. Division into equal organisms.

transverse division, which, indeed, I regarded as probably the true mode of propagation. According to this conception the attenuated parts described would represent the points of separation. Further studies have supplied me with unmistakable evidence of transverse division. The length of these specimens of pallida was unusually great, and in the course of one spiral three or even four thinner and straighter portions were encountered. These specimens were found in preparations in which the large numbers present indicated active multiplication. The attenuations occurred at intervals of six to eight curves. It is difficult to account for this appearance upon any other supposition than transverse division. Admitting this as the probable mode of division, it should be said that not infrequently the product is greater than two individuals at one division; I have seen a dividing spiral which apparently was yielding four individuals.

Koch[2] has just published an account of the study of the spirochætæ of recurring African fever and his figures show unmistakable evidence of transverse division.

These figures show so great a similarity to those which I obtained from Spirochæta pallida that the two organisms may, I think, be regarded as multiplying in the same manner. Other points of similarity between the recurrens and the pale spirochætæ are the occurrence of thick individuals and the tendency to form masses where the organisms are sufficiently abundant and the medium suitable.

It must, however, be considered as still a question whether some of the thick and uniform types of pallida represent two accidentally intertwined individuals. It does not seem impossible that conjugation may be going on in these forms. And if this were true, then the intertwinings and the zooglea-like formations would not represent accidental conditions, but unions looking to renewal of life by the fusion of two elements. The remaining possibility, which is not to be excluded, is that the thick forms are involutions. I have seen undoubted examples of involutions in short, thick pallida.

I am deeply indebted to Professor E. K. Dunham for many helpful suggestions which led me to definite conclusions regarding the mode of division of the pallida. Further work is now being carried on largely along lines suggested by Professor Dunham, who has taken an active interest in this work and has placed the facilities of the laboratory at my disposal. I wish also to express my gratitude to the Dean of the College, Professor Egbert Le Fevre. While the number of gentlemen who have favored me with cases, syphilitic and others, upon which my work is based, is too great to permit of individual mention, I do not wish to fail to acknowledge my special indebtedness to Dr. Flexner of the Rockefeller Institute who has been ever ready to help and suggest, to Professor Fordyce and his staff of the Dermatological Department, and to Professor van der Poel and his staff of the Department of Genito-Urinary Diseases. Had it not been for the courtesy of these gentlemen, I should have been without the most valuable material.

[2] Koch, *Berl. klin. Wochenschr.*, 1906, xliii, 185.

EXPLANATION OF PLATES

PLATE XXI.

(All Spirochæta pallida.)

Fig. 1.—(*a*) Division into three individuals.
(*b*) " " two "

Fig. 2.—Division into three individuals, red blood corpuscles in the field.

Fig. 3.—(*a*) Very long pallida; atypical curves.
(*b*) Long pallida, partly double and thicker than normal.
(*c,c,c*) Typical specimens of pallida.

Fig. 4.—(*a*) Single organism, abnormally thick.
(*b*) Division into two organisms.
(*c*) Typical pallida.

Fig. 5.—Massing of spirochætæ; some blood corpuscles in the field.

All figures from moist papules; original amplification × 2200; reduced $\frac{1}{3}$.

PLATE XXII.

Fig. 1.—(*a*) Spirochæta pallida; close intertwining of two individuals.
(*b*) Long pallida; only partly shown. (× 1800.)

Fig. 2.— Very long pallida; curves partly atypical; evidence of transverse division; a second short organism intertwined at one end. Red corpuscle near center. (× 1800.)

Fig. 3.—Spirochæta pallida; division into three. (× 1800.)

Fig. 4.—(*a* and *b*) Typical pallida.
(*c* and *d*) Atypical curves in pallida.
(*e*) Thick pallida apparently splitting into three.
(*f*) Two pallida spirochætæ, partly so closely coiled as to resemble thick part of *e*. (× 1800.)

Fig. 5.—Apparently single pallida, attenuated at each end. (× 2200.)

Fig. 6.—Pallida with *straight* central body. (× 2200.)

Fig. 7.—Spirochæta refringens with membrane and blunt projection at one end which stains blue while the body is deep purple in color. (× 1800.)

Fig. 8.—(*a*) Spirochæta refringens; blunt projection as in Fig. 7.
(*b*) Spirochæta plicatilis, probably two individuals closely applied. (× 1300 approx.)

Fig. 9.—Spirochæta pallida; three double organisms of which A is irregularly intertwined; B and C show at points indicated by arrows perfect apposition of coils. (× 1800.)

Fig. 10.—(A) Spirochæta pallida, 7 coils.
(B) " " 29 "
(× 1800.)

Figs. 1, 2, 3, 4, reduced $\frac{1}{3}$.

Figs. 1, 2, 3, 4, 5, 9, from a moist papule under breast.

Figs. 7 and 8 from mucous patch.

Fig. 10 from a chancre of lip.

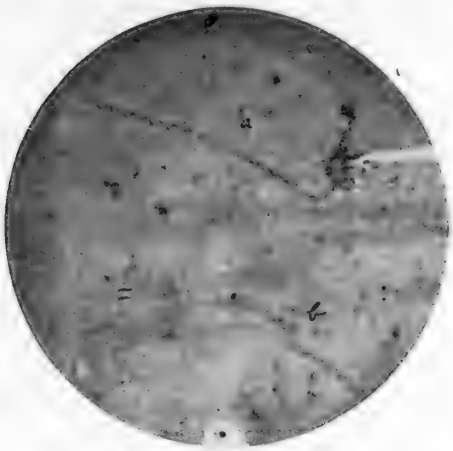


Fig. 1.

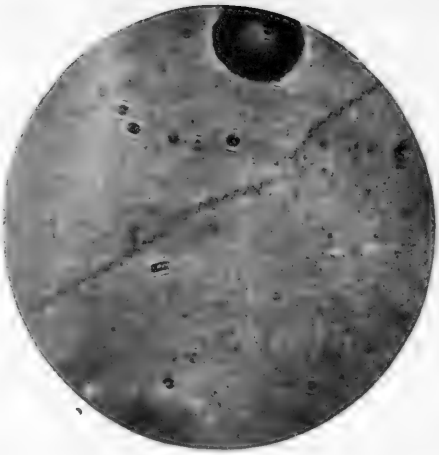


Fig. 2.

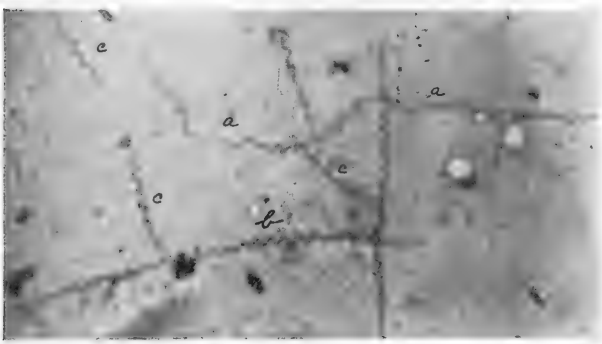
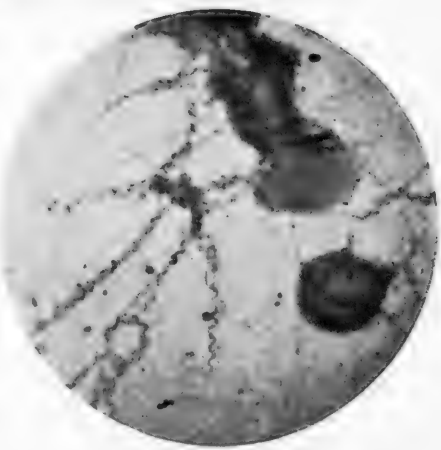


Fig. 3.

Fig. 4.



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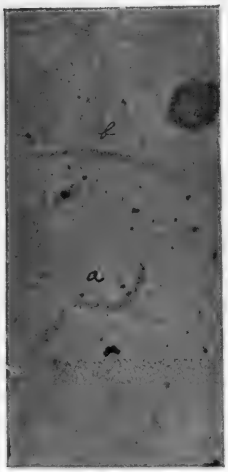


Fig. 1.

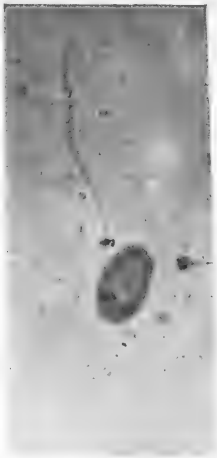


Fig. 2.

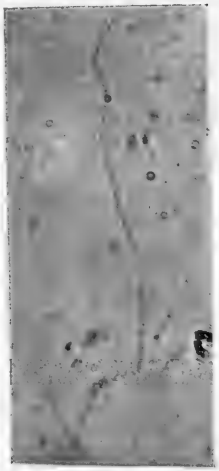
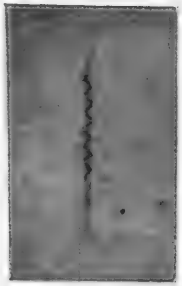


Fig. 3.



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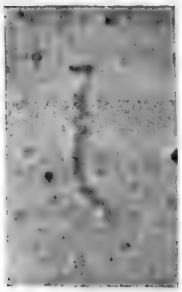
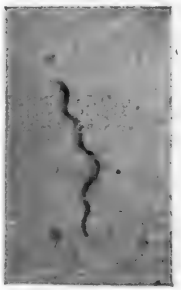


Fig. 6.

Fig. 4.



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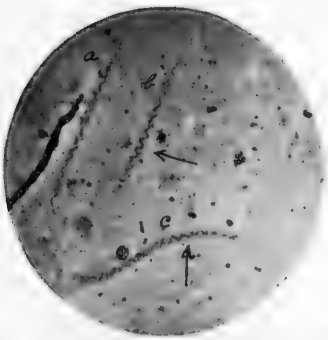

Fig. 9.

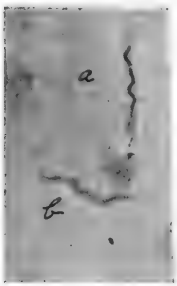

Fig. 8.

Fig. 10.

ON GLYCYLPROLIN ANHYDRIDE OBTAINED ON TRYPTIC DIGESTION OF GELATINE.

By P. A. LEVENE and W. A. BEATTY.

(*From the Rockefeller Institute for Medical Research, New York.*)

In a previous communication on tryptic digestion of gelatine one of us (Levene) reported the finding of a substance having the composition $C_7H_{10}N_2O_2$.[1] The mode of separation of this substance was fully described in an article published by G. Wallace and one of us (Levene [2]). In the previous publications the supposition was expressed that the substance might be a precursor of prolin. Indeed on further cleavage with mineral acid the substance $C_7H_{10}N_2O_2$ decomposed into prolin and glycocoll, thus showing that it is a dipeptid, prolylglycin anhydride—$C_5H_9NO_2 + C_2H_5NO_2 - 2H_2O = C_7H_{10}N_2O_2$.

One and five tenths grams of the air dry substance was heated for five hours with strong hydrochloric acid in a sealed tube at 150° C. The product of the reaction was taken up in water and evaporated to dryness under diminished pressure, and the residue again dissolved in water, and evaporated. This operation was repeated three times. The remaining hydrochloric acid was removed by means of silver sulphate, and the sulphuric acid by means of barium hydroxide, and the filtrate from the barium sulphate was evaporated to dryness. The residue was crystalline in nature, possessed a very sweet taste, formed with copper oxide a salt, gave rise to pyrrol on sublimation, and, on heating, gave the odor of pyrolidin. Thus the presence of prolin in the residue was made very probable. The presence of glycocoll was demonstrated by the formation of the typical picrate described by one of us (Levene).

The separation of the two aminoacids was accomplished by means of boiling methyl alcohol. The mixture was repeatedly extracted with methyl alcohol until the residue no longer gave on

[1] *Journal of Exper. Med.*, 1906, viii, 180.
[2] *Zeitschr. f. physiol Chem.*, 1906, xlvii., 143.

sublimation the pyrrol test. The substance gave a bright pink sublimate which is characteristic for glycocoll formed a picrate resembling that of glycocoll, and possessed a very sweet taste. On rapid heating it turned brown and began to melt at 240° C., and decomposed at 250° C. (An Anschutz thermometer was used for the experiment.) The substance had the following composition:

0.0881 gram of the substance gave on combustion 14.4 c.c. nitrogen (over 50% KOH) at T° = 18.5° C and p = 747 m.m.

For $C_2H_5NO_2$ the quantities are as follows:

Calculated.	Found.
N = 18.66%	18.87%

Thus the substance was glycocoll. The yield of it was 0.350 gr.

The methyl alcohol extract was concentrated under diminished pressure and then evaporated nearly to dryness on a water bath. It was then taken up in absolute alcohol, filtered, and dried. The substance had all the properties of prolin. Its melting point was 205° C.

0.185 gram of the substance gave on combustion 20.8 c.c. nitrogen (over 50% KOH) at T° = 22° C. and p = 760 m.m.: N = 13.03%.

For $C_5H_9NO_2$ the quantities are as follows:

Calculated.	Found.
N = 12.17%	13.03%

Thus this sample consisted of prolin still containing some glycocoll.

The alcoholic mother liquid was allowed to evaporate over sulphuric acid in vacuum desiccator. A crystalline deposit was formed. It was filtered, washed with alcohol and ether, dried, and analyzed:

0.1356 gr. of the substance gave on combustion 0.2600 gr. CO_2. The water absorption tube was lost through an accident. For $C_5H_9NO_2$:

Calculated.	Found.
C = 52.17%	52.28%

0.1037 gr. of the substance gave on combustion 0.1995 gr. CO_2 and 0.740 gr. H_2O. For $C_5H_9NO_2$:

Calculated.	Found.
C = 52.17%	52.46%
H = 7.82%	7.92%

This finding furnishes conclusive evidence, lacking up to now, that prolin is a primary component of the proteid molecule.

ON THE PHOSPHOTUNGSTATES OF CERTAIN AMINOACIDS.

By P. A. LEVENE and W. A. BEATTY.

(*From the Rockefeller Institute for Medical Research, New York.*)

In a communication to the Chemical Society[1] one of us reported on the property of aminoacids to form insoluble phosphotungstates. In a later publication[2] the writers showed the degree of precipitability of aminoacids by means of phosphotungstic acid, and stated that a separation of aminoacids may be accomplished by means of this reagent. The attempt was made to apply the method to the study of the acids resulting from the hydrolysis of gelatine by means of mineral acids. Unfortunately, through an accident in the laboratory, the greater part of the material for this study was lost, and hence in this place there will be reported a method of the separation of oxyprolin and of an acid having the composition $C_7H_{14}N_2O_2$.

Oxyprolin.—This communication is prompted partly by a statement made by Emil Fischer in a recent address delivered to the German Chemical Society in Berlin.[3] Fischer points out that considerable difficulty is experienced in obtaining oxyprolin from the mixture of the products resulting from hydrolysis of proteids. The process employed by Fischer consisted in removing most of the aminoacids first by esterification, then by crystallization, and finally by means of phosphotungstic acid. From the mother liquid, remaining after all these precipitations, oxyprolin was separated.

We have observed that oxyprolin can be isolated as a phosphotungstate without great difficulty. It precipitates in the form of an oil which crystallizes on standing and is soluble in an excess of the reagent. Its separation was accomplished in the following manner:

[1] Jan. 5, 1906; see *Science*, 1906, p. 347.

[2] *Zeitschr. f. physiol. Chem.*, 1906, xlvii, 149.

[3] *Berichte d. deut. Chem. Gesellschaft*, 1906, xxxix, 597.

The product of hydrolysis (after the mineral acid has been removed in the usual manner) was concentrated to a thick syrup, acidulated with sulphuric acid, and treated with a concentrated solution of phosphotungstic acid (4 to 1) so long as an oily precipitate was formed. The supernatant liquid was decanted and the oil treated with an excess of the phosphotungstic acid solution until the oily character of the precipitate disappeared. A part of the oil passed into solution and another part formed a precipitate with the excess of the reagent. The soluble part contained the oxyprolin. On concentration of the solution (after the phosphotungstic acid was removed) leucin first crystallized. The mother liquid from this acid was further concentrated, after which a second precipitate formed. This substance was removed by filtration, washed with alcohol and ether, dried, and analyzed. The crude substance had the following composition:

0.1271 gram of the substance gave on combustion 0.2242 gram CO_2 and 0.1033 gram of H_2O; C = 48.09%, H = 9.03%.

It was then redissolved in little water and two volumes of absolute alcohol were added. A precipitate appeared consisting of typical crystals of oxyprolin. The crystals tasted very sweet and formed pyrrol on sublimation. The melting point was at 270° C.; the composition was as follows:

0.1342 gram of the substance gave on combustion 0.2266 gram CO_2 and 0.0843 gram H_2O. Hence for $C_5H_9NO_3$

Calculated.	Found.
C = 45.80%	46.05%
H = 6.87%	6.9%

From this analysis the substance is oxyprolin.

On the Substance $C_7H_{14}N_2O_5$.—This substance was obtained from the fraction of the products of hydrolysis consisting chiefly of glycocoll. It had been stated in a previous communication that glycocoll and alanin under favorable conditions may be separated nearly quantitatively by means of phosphotungstic acid. These phosphotungstates possess typical crystalline forms and are slightly soluble in an excess of the reagent. A phosphotungstate of this nature was obtained from the products of hydrolysis of gelatine. The glycocoll was removed in the form of

a picrate. From the mother liquid of the glycocoll picrate, the picric acid was removed in the usual manner, and the solution concentrated to a very small volume and then treated with an excess of absolute alcohol. An oily sediment was formed which on standing was beaded with granular crystalline deposits, and similar deposits were formed on the walls of the vessel. The substance possessed a very sweet taste, gave on sublimation pyrrol, had a melting point of 236° C., and yielded the following composition:

0.1854 gram of the substance gave on combustion 0.2772 gram CO_2 and 0.1196 gram H_2O; C = 40.76%, H = 7.17%.

In another experiment the substance was obtained in an identical manner and the attempt to purify it by recrystallization was made. For that purpose it was redissolved in little water and treated with alcohol. But as in the course of twenty-four hours no crystal formation appeared, a great excess of alcohol was added. An amorphous precipitate was formed, having a melting point of 232° C. and the following composition:

0.2468 gram of the substance gave on combustion 0.3710 gram CO_2 and 0.1880 gram H_2O; C = 40.99%, H = 7.13%.

In a third experiment the substance obtained in the same manner was not absolutely free from glycocoll and it contained 37.00% of carbon and 8.00% of hydrogen. This substance was then extracted with a great excess of methyl alcohol. A part passed into solution. The insoluble part was pure glycocoll. It was then attempted to purify the bodies of the first and the second experiment by means of methyl alcohol. It was, however, found that they were completely dissolved in an excess of the reagent. The combined methyl alcoholic solutions were concentrated to a small volume and precipitated with aceton. The precipitate thus formed was amorphous and not absolutely white. It was therefore extracted with a very small quantity of boiling methyl alcohol, washed with alcohol and ether, dried in the xylol bath, and analyzed. It had the following composition:

0.1682 gram of the substance gave on combustion 0.2469 gram CO_2 and 0.1077 gram of H_2O; C = 40.75%; H = 7.12.%

0.149 gram of the substance gave on combustion 17.5 c.c. (over 50% KOH) Nitrogen at T° = 22.5° C. and p = 775 m.m.: N = 13.73%.

The remaining part of the substance was dissolved in a large excess of boiling absolute alcohol. It all went into solution leaving practically no residue. The excess of alcohol was then removed under diminished pressure and the solution further concentrated on a water bath. A crystalline deposit consisting of prismatic needles was formed. In an aqueous solution it reacted acid to litmus, possessed a sweet taste, and had a melting point of 236°–240° C. On sublimation pyrrol was given. An analysis gave the following composition:

0.1214 gr. of the substance gave on combustion 15.00 c.c. nitrogen (over 50% KOH) at T°=25° C. and p=763 m.m.; N=14.25%. For $C_7H_{14}N_2O_5$:

Calculated.	Found.			
	Sample I.	Sample II.	Sample III.	Sample IV.
C = 40.75%	40.76%	40.99%	40.75%	
H = 6.8%	7.17%	7.13%	7.12%	
N = 13.54%	13.33%		13.73%	14.25%

Thus it was impossible by means of the various methods to alter the composition of the substance, and it is, therefore, probable, that the analyzed body was not a mixture but an individual substance of the composition $C_7H_{14}N_2O_5$. The substance may perhaps be a homologue of Fischer's acid $C_{12}H_{24}N_2O_5$.